INSIGHTS INTO THE BIOSYNTHESIS OF THE LANTIBIOTIC MUTACIN 1140

A Dissertation

by

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ABSTRACT

The rise of antibiotic-resistant bacteria poses a large problem for healthcare systems across the world. This problem is further exacerbated by the lack of development in new types of antibiotics. The CDC has identified antibiotic development as one of the key solutions in combating antibiotic resistance. Lantibiotics, a class of posttranslationally modified (PTM) peptide antibiotics, are promising candidates for antibiotic development. Lantibiotics are characterized by the presence of lanthionine rings and dehydrated residues. Mutacin 1140, produced by *Streptococcus mutans* JH1140, is a lantibiotic which has shown promising activity against a variety of Gram-positive pathogens. Although lantibiotics, such as mutacin 1140 show promise, they are still hampered by the lack of development. Furthering the understanding of lantibiotic biosynthesis is crucial for promoting the development of lantibiotics.

Lantibiotic biosynthesis requires a leader peptide for efficient posttranslational modification, which is then cleaved off to produce the full lantibiotic. A novel four amino acid EDLF motif was found to be important for biosynthesis in Mutacin 1140. Additionally, I have shown that a second cleavage occurs 8 amino acids upstream of the defined MutP cleavage site, and that this cleavage event is dependent upon position and not sequence. I sought to determine the importance of different PTM's in the formation for the coordination of PTMs and transport of mutacin 1140. Deletion of the lanthionine rings show that lanthionine ring formation affects other PTM's, and that it is important for transport out of the cytoplasm.

The role of the unique S-[(Z)-2-aminovinyl]-D-cysteine (AviCys) residue found in mutacin 1140 is poorly understood. Removal of the C-terminal carboxyl group increases the antibiotic's affinity to its molecular target lipid II. A carboxyl variant of mutacin 1140 does not affect the other PTM’s and is important for activity. This variant is agreeable to the chemical addition of a wide variety of substrates, producing novel variants of mutacin 1140 with restored activity class of lantibiotics that can be screened for improvements in drug development. The advancements made in understanding the mutacin 1140 biosynthesis pathway will help in developing mutacin 1140 as a tool to help combat the problem of antibiotic resistance.

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NOMENCLATURE

AviCys	S-[(Z)-2-aminovinyl]-D-cysteine
DAP	Diamino pimelate
Dha	2,3-didehydroalanine
Dhb	2,3-didehydrobutyrine
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
FPLC	Fast protein liquid chromatography
LD_{50}	50% of lethal concentration
HOAt	1-Hydroxy-7-azabenzotriazole
HPLC	High performance liquid chromatography
MALDI-MS	Matrix-assisted laser desorption/ionization - Mass spectrometry
MDR	Multi-drug resistant
MIC	Minimum inhibitory concentration
MIC_{50}	50% of minimum inhibitory concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
NRPS	Nonribosomal peptide synthetases
PTM	Postranslational modification
SOPMA	Self-optimized prediction method with alignment
SPPS	Solid-phase peptide synthesis
VREF	Vancomycin-resistant *Enterococcus faecium*

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CHAPTER I:

LITERATURE REVIEW: MULTIPRONGED APPROACH FOR ENGINEERING NOVEL PEPTIDE ANALOGS OF EXISTING LANTIBIOTICS[*]

I.1. Introduction

The emergence of multi drug-resistant (MDR) strains of bacteria poses a significant threat to public health and has cost the healthcare system billions of dollars annually (1-3). MDR bacteria are one of the leading causes of hospital acquired infections and have led to an increase in patient deaths worldwide (4, 5). This problem is further exacerbated by the lack of development of new antibiotics that can help combat MDR bacteria (6-8). Lantibiotics, a class of ribosomally-produced and posttranslationally-modified peptide antibiotics, is one such class of antibiotics that can complement current antibiotics for the treatment of Gram-positive MDR infections (9). Lantibiotics have been shown to have activity against drug-resistant Gram-positive pathogens, such as methicillin resistant *Staphylococcus aureus* (MRSA) (10-12). Furthermore, lantibiotics have been shown to have promising efficacy and pharmacokinetics in animal models (13-16). Most lantibiotics target lipid II, an essential component of cell wall biosynthesis. Lantibiotics, such as nisin and mutacin 1140, form a cage like structure around the pyrophosphate moiety of lipid II, the essential nature of this target leads to decreased chances in developing resistance (17). Together with its mode of action and broad spectrum of activity against Gram-positive bacteria,

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[*] Reprinted with permissions from Multipronged approach for engineering novel peptide analogues of existing lantibiotics by Jerome Escano and James Smith. 2015. *Expert Opinion on Drug Discovery*. 10:8. Copyright [2015] by Escano et al 2015 DOI: 10.1517/17460441.2015.1049527

lantibiotics have tremendous potential for their development as a therapeutic against MDR infections. Furthermore, the development of lantibiotic producing microbial strains as probiotics is another application of lantibiotics (18), such as the use of *Streptococcus salivarius* and *Streptococcus muta*ns strains (19, 20). *Streptococcus mutans*, producer of mutacin 1140, is used in a technology referred to as replacement therapy to prevent dental caries. This therapy is aimed at reducing disease causing oral bacteria by supplementing the microbial niche with a non-disease causing genetically engineered strain of *S. mutans* (21, 22).

Nisin was the first lantibiotic to have its covalent structure characterized. Since the structural characterization of nisin, a vast diversity of lantibiotic structures has been reported (23-27). The lantibiotic core peptide generally contains several posttranslational modifications (PTMs) and is aptly named due to the presence of lanthionine residues (Figure 1.1A). All lantibiotics prior to cyclization, possess 2,3-didehydroalanine (Dha) and 2,3-didehydrobutyrine (Dhb), which are dehydrated serines and threonines, respectively. During cyclization, Dha or Dhb residues are further modified by forming thioether linkages with neighboring cysteines to form lanthionine or methyllanthionine rings, respectively (Figure 1.1A). Individual types of lantibiotics can have further modifications, such as the AviCys residue found in class I epidermin group of lantibiotics, D-alanine in lacticin 3147, and β-hydroxy aspartate in cinnamycin (28-30). The vast majority of lantibiotics have a similar biosynthetic gene loci and nomenclature. Lantibiotics have traditionally been subdivided into three major classes, class I lantibiotics which use both LanB and LanC for lanthionine synthesis, class II

lantibiotics which use bifunctional enzyme LanM for lanthionine synthesis, and class III lantibiotics which include all lantibiotics that do not have any antibacterial function but rather a regulatory function. Recently, a new class of lantibiotics that utilizes a unique synthetase, LanL, has been further characterized as class IV lantibiotics (31). The vast majority of lantibiotics fall within the class I and class II classification. In general, LanA denotes the unmodified core peptide attached to its leader peptide. In class I lantibiotics, LanB is a dehydratase that dehydrates serines and threonines, and LanC is the cyclase which helps form lanthionine and methyllanthionine rings (Figure 1.1A). In class II lantibiotics, a single gene, *lanM,* expresses a single bifunctional enzyme that performs the function of both LanB and LanC. LanP is a serine protease which cleaves off the leader peptide after transport by the ABC transporter LanT in class I lantibiotics. In class II lanbiotics, LanT is a bifunctional protein with an additional protease domain. The proteolytic release of the core peptide from the leader peptide triggers the antibacterial activity of the core peptide (32). Most lantibiotics have the producer immunity proteins LanFEG, which have also been shown to act as a secondary transport system. In general most lantibiotic biosynthetic gene clusters contain a two-component histidine kinase regulatory system, LanR and LanK (33). Additionally, some lantibiotic systems, such as gallidermin, have an additional immunity gene, LanI. Further studies aimed at increasing our understanding of the genetics of lantibiotic biosynthesis along with an in depth understanding of the limitations in lantibiotic structural diversity will promote development of this class of interesting antimicrobials for therapeutic use.

Although lantibiotics are a promising class of novel therapeutics, development of lantibiotics such as nisin, has been hampered by limitations in production, low solubility, and the susceptibility of lanthionines to oxidation (34-36). Creating novel variants from existing lantibiotics can help solve this problem. Mainly three different strategies have been utilized to produce novel lantibiotics through engineering. (i) *In vivo* engineering involves creating lantibiotic variants that have increased activity by manipulating the biosynthetic gene cluster (37, 38). This strategy can also help engineer lantibiotic producing strains of bacteria to synthesize significantly more antibiotic (39-41). (ii) Chemical synthesis provides another avenue toward the production of lantibiotics. This strategy opens a wide array of choices of producing novel lantibiotics with synthetic modifications and non-proteinogenic amino acids (42). (iii) *In vitro* engineering/semi-synthetic methods offer the advantage of producing semi-synthetic lantibiotics that retain the stereochemistry of the natural product, while being able to enhance the bioactivity at site specific locations (43). This strategy encompasses several methods, such as the incorporation of non-proteinogenic amino acids and site specific additions of functional groups to the mature lantibiotic (44). These three strategies show the flexibility and promise the lantibiotic field can offer in terms of producing novel and effective therapeutics to treat MDR infections. This review further explores different studies that have utilized these three strategies in generating novel lantibiotics.

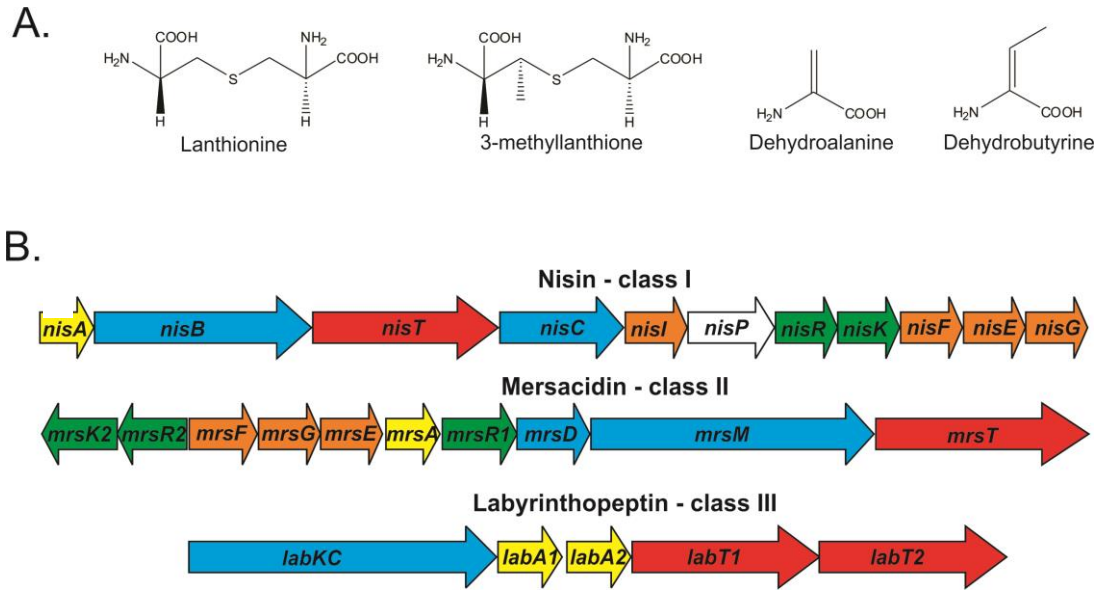

Figure 1.1 Schematic of common lantibiotic structures and biosynthesis genes. **A**) Structures of common posttranslational modifications found in lantibiotics. **B**) Biosynthetic gene loci for different classes of lantibiotics. Orange genes indicate genes involved immunity. Green genes indicate genes involved in regulation. Yellow designates the gene for the lantibiotic product. Blue are genes involved in amino acid dehydration and lanthionine synthesis. Red encodes for the transporter. White encodes for the lantibiotic specific protease.

I.2. *in vivo* engineering of lantibiotics

I.2.1 *Class I lantibiotics*

In bacterial systems that have the tools developed for genetic manipulation, enhancing the activity or production of lantibiotics by amino acid substitution provides an effective and relatively straight-forward method to create novel structural variants. In the past 10 years there have been a number of genetic engineering studies which have successfully been able to produce lantibiotic variants with increased activity. Nisin-producing *Lactococcus lactis* has been successfully used for over 50 years in the dairy industry to prevent unwanted bacterial contamination (45). The class I lantibiotic, nisin, is responsible for its utility as an organic preservative and this application in food microbiology has promoted the study of nisin (46). Therefore, there are several mutagenesis studies leading to the production of novel variants of nisin. A substitution of threonine in position 2 for a serine increased activity of nisin against multiple strains tested (Figure 1.2A) (47). This mutation also led to the dehydration of this serine indicating promiscuity of the dehydration for some amino acid substitutions. Some amino acid substitution mutants, such as the substitution of three amino acids in ring A had shown enhanced activity (Figure 1.2A) (48). Furthermore, the VFG variant had decreased activity against other indicator strains but had increased activity against the producing strain, indicating loss of host immunity protection. This is an important finding given that resistance mechanisms found in clinical isolates often share enzymatic resistance mechanisms found in producing strains. The study demonstrates that it is possible to modify the lantibiotic peptide in a manner that may prevent the selection and

clinical development of producer strain resistance. In some class I lantibiotics, such as nisin and mutacin 1140, rings A and B are essential for binding to the pyrophosphate moiety of its cellular target lipid II (49-52). Initial binding to lipid II's pyrophosphate moiety is through the NH backbone of rings A and B, thus amino acid substitutions in Rings A and B could potentially stabilize or increase affinity to lipid II. A saturation mutagenesis study on nisin led to the discovery of additional variants with enhanced bioactivity. In the hinge region of nisin, between ring B and C, the substitution K12A had shown an increase in activity (53). Single amino acid substitutions in another nisin hinge region between rings C and D also enhanced activity against multiple strains tested (Figure 1.2A) (54-56). Interestingly, there is a structural diversity within the hinge region of other natural peptide analogs of nisin. Thus, it was expected that structural variants within the hinge region could be isolated. The identification of natural variants of lantibiotics is important for identifying structural regions that are amenable to amino acid substitutions. The mutation S29G in nisin had a 2-fold increase in MIC against selected pathogens (57). In the related class I peptide mutacin 1140, a single amino acid substitution at W4A in ring A had a significant increase in activity against several Gram-positive strains tested, including the pathogenic strain *Clostridium difficile* (Figure 1.2A) (40). The R13D mutant in the hinge region of mutacin 1140 had the largest zone of inhibition activity, but the purified product had a significant reduction in activity. Thus, this mutation was expected to have promoted the production of the R13D variant compared to the wild type variant (40). The substitution to an aspartic acid may have interfered with the regulation of mutacin 1140 production or possibly increased the

peptide's stability. Class II lantibiotics are structurally distinct from class I lantibiotics and the structural regions that promote bioactivity are also uniquely different.

I.2.2 *Class II lantibiotics*

Aside from utilizing the bifunctional enzyme, LanM, class II lantibiotics generally have a more globular structure compared to class I lantibiotics (33). In the past 6 years, multiple studies have been able to produce enhanced variants of existing class II lantibiotics. The most well-known and studied of class II lantibiotics is mersacidin. It is naturally produced by *Bacillus sp.* HILY-84,54728, but has had its biosynthesis genes moved into the naturally competent *Bacillus amyloliquefaciens* FZB42 strain (58, 59). In a saturation mutagenesis study for mersacidin, only the mutation F3W increased activity against the medically important bacteria *S. aureus* and *S. epidermidis* (Figure 1.2B). Other mutations in mersacidin had enhanced activity, but only against a few of the bacterial strains tested (60). Saturation mutagenesis was performed on a similar lantibiotic, actagardine A, produced by *Actinoplanes garbadinensis* ATCC 31049. One mutant, V15F had increased activity against some pathogenic strains, such as *Streptococcus pneumoniae* (Figure 1.2B) (61). Nukacin-ISK1, produced by *Staphylococcus warneri* ISK1, is another class II lantibiotic that has been bioengineered to produce enhanced variants. Two single substitution mutants, D13E and V21I were able to enhance activity (Figure 1.2B) (39). The reason for the increase in bioactivity is not readily apparent, given the similarity between the amino acid substitutions. The aspartic acid residue at position 13 is located in ring A of nukacin-ISK1. The mutation to

a similarly charged glutamic acid may promote access or affinity to lipid II. In both class II lantibiotics, mersacidin and actagardine, a negatively charged residue at this position is conserved, indicating importance of this negatively charged residue for class II lantibiotics (62). Zendo et al hypothesized that the substitution of valine at position 21 to a bulkier isoleucine may limit the peptide rotation when bound to target, thus, increasing its affinity. Mutations in the histidine at position 15 did not increase activity for the purified nukacin-ISK1, but had increased lantibiotic production by almost 4-fold compared to wild type (39). The two component lantibiotic lichenicidin, normally produced by *Bacillus licheniformis* DSM13, had its biosynthesis pathway fully reconstituted into a Gram-negative host *E. coli*. The transfer of the biosynthetic pathway promoted mutagenesis studies that enabled the characterization of essential structural elements within the two component system (63). An alanine scanning mutagenesis study on the two component lantibiotic lacticin 3147, produced by *L. lactis* subsp. lactis DPC3147, was performed and only one of the substitutions increased activity but has not been tested against multiple strains (Figures 2C) (64). Producing enhanced variants of two-component lantibiotics is much more challenging given that two lantibiotic peptides work together in synergy. Recently, a single variant in lacticin 3147 Ltnα in which the histidine at position 23 was substituted with a serine had an increase in activity against a pathogenic strain of *Staphylococcus aureus* (65).

I.2.3 *Increasing yield through in vivo engineering*

Low fermentation and isolation yields for many lantibiotics has hampered the development of lantibiotics as a feasible therapeutic. *In vivo* engineering of the producing strains offers a method to increase yields of lantibiotics. Amino acid substitutions have been shown to increase production of some lantibiotics. Mutations in the histidine at position 15 of nukacin-ISK1 did not increase activity for the purified product, but had increased lantibiotic production by almost 4-fold compared to wild-type. Similarly, the mutation R13D in mutacin 1140 reduced activity, but had significantly increased yields. Panke et al used a modified strain of *Staphylococcus gallinarum* Tü3928 along with stress conditions to induce higher yields of gallidermin. This modified *S. gallinarium* strain produced a variant of gallidermin with the leader peptide attached, which prevented self-toxicity of the mature lantibiotic (41, 66). In addition, the subjection of some lantibiotic producing strains to stressful conditions can increase production yields compared to normal growth conditions (67-70). Overexpression of nisin and PTM enzymes using inducible promoters has supported studies on nisin. The nisin induced expression system has been used for over a decade for the production of nisin (71). Likewise, production of nisin was significantly increased compared to the normal producing strain by the optimization of a nisin overexpression cassette (72). Conversely, some lantibiotics are difficult to produce and genetically manipulate due to the nature of the producing strain. Lantibiotics, such as nukacin-SK1 and mersacidin have been heterologously produced in other bacterial species, not only to increase yields but to facilitate mutagenesis experiments (39, 58, 73).

To further expand lantibiotic production tools, heterologous expression in *E. coli* has been attempted for various class II lantibiotics (63, 74-76). A successful example of heterologous expression in *E. coli* is for the lantibiotic lichenicidin. Studies that promote the production and isolation of lantibiotics in combination with studies that enhance their bactericidal activity will further the development of lantibiotics for commercial applications.

In vivo engineering of lantibiotics has produced variants with enhanced activity or production. This method is relatively simple and efficient way to create large libraries of lantibiotic analog. However, *in vivo* engineering has its limits given that the PTM enzymes generally do not accommodate all amino acid substitutions, thus, not all positions can be modified. This is generally observed at amino acid positions that disrupt lanthionine formation, ring size, or lanthionine location (77). Furthermore, the lantibiotic producer strain usually has some limitations, such as low yields of production and lack of genetic manipulation tools. Most *in vivo* engineering studies focus on increases in activity of the purified lantibiotic variants, but fail to report notable changes in yields. Alternative methods, such as chemical synthesis can help overcome these limitations.

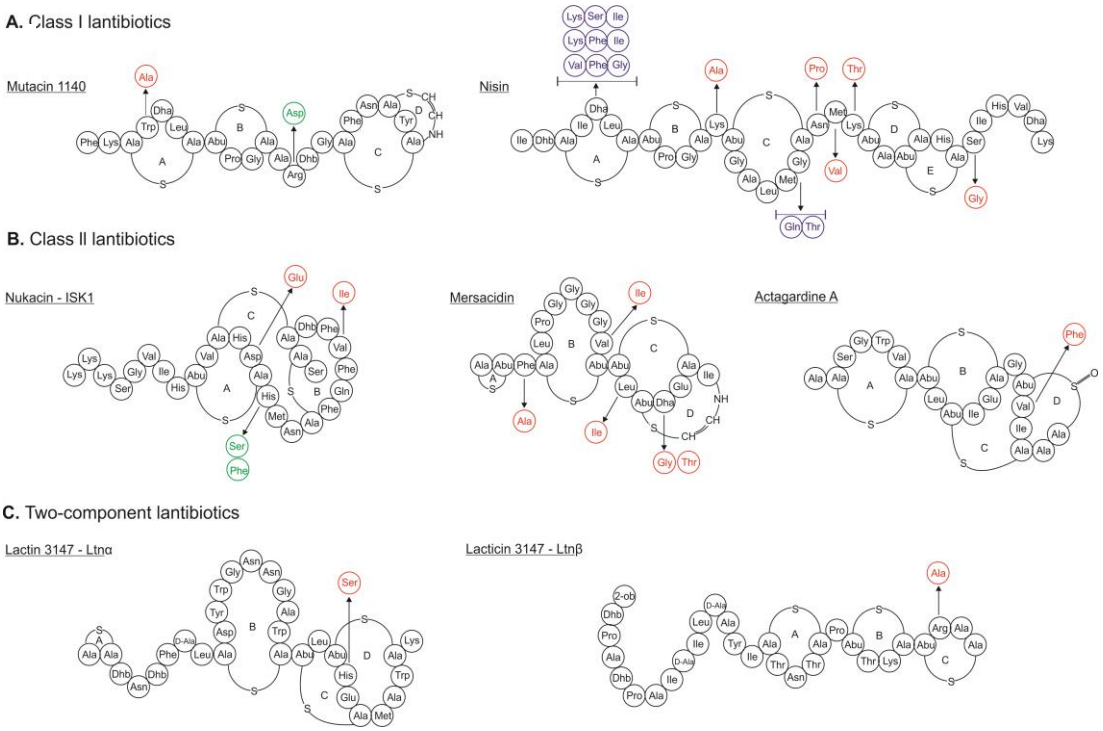

Figure 1.2 Structures and modifications of lantibiotics enhanced through *in vivo* engineering, **A**) Class I lantibiotics; Mutacin 1140 and Nisin (40, 53), **B**) Class II lantibiotics; Nukacin – ISK1, Mersacidin and Actagardine (39, 60, 61), **C**) Two-component lantibiotics, Lacticin 3147 – Ltnα ans Lacticin 3147 – Ltnβ (64, 65). Unusual amino acids are labeled as such: Dha: 2,3-didehydroalanine, Dhb, 2,3-didehydrobutyrine, Ala-S-Ala: lanthionine ring, Ala-S-Abu: methyllanthionine ring, D-Ala: D-alanine, 2-ob: 2-oxobutyrate. Arrows designate amino acid substitution within the native lantibiotic structure. Amino acid substitutions colored in red resulted in an increase in activity. Substitutions in green resulted in an increase in lantibiotic production. Substitutions in blue designate multiple amino acids substitutions within the core peptide that resulted in an increase in activity.

I.3. Chemical synthesis of lantibiotics

I.3.1 *Synthesis of native lanthionines*

Chemical synthesis of lantibiotics bypasses the limitations of the modification machinery, and allows for greater variety of analog to be produced. Furthermore, this allows for the incorporation of non-proteinogenic amino acids, which can promote the generation of large structural libraries that can be screened for improvements in activity. Although promising, chemical synthesis of lantibiotics poses a unique challenge to chemists due to the extent of posttranslational modifications found within lantibiotic peptides. For a long period of time, a solution chemical synthesis route of nisin was the only successful instance of producing a full length lantibiotic (78). This analog of nisin had the same activity as natural nisin against a broad range of Gram-positive bacteria, including some pathogenic strains. However, the overall yield from this approach was low and the method did not afford a viable approach for production. Due to recent advances in solid phase peptide synthesis (SPPS), many other lantibiotics have been chemically synthesized. The synthesis of orthogonally protected methyl/lanthionine rings has allowed for tremendous advances in lantibiotic chemical synthesis (Figure 1.3A) (79-81).

SPPS of lantibiotics was first limited to lantibiotics containing non-overlapping lanthionine rings. Over a decade after the synthesis of nisin by a solution chemical synthesis route, lactocin S was the first full length lantibiotic to be produced using SPPS (82). This synthetic analog had similar activity as the natural variant in a spot lawn assay against *Pediococcus acidilactici* Pac 1.0. Furthermore, a variant of lactocin S with the

non-proteinogenic amino acid norleucine at the methionine 12 position had enhanced activity (Figure 1.3B) (83). The substitution of methionine for a norleucine was done to prevent oxidation at this position. A challenge for many other lantibiotics is the presence of overlapping lanthionine rings, as is the case for rings DE in nisin. The synthesis of additional building blocks with differentially protected orthogonal lanthionine groups helped overcome the restriction of synthesizing lantibiotics containing overlapping lanthionine rings (Figure 1.2) (84). In the two component lantibiotic lacticin 3147, both peptide components have been chemically synthesized (84). This study was the first instance of using Fmoc SPPS technology in creating overlapping lanthionine rings. When paired with their natural counterparts, the synthetic lacticin 3147 components had virtually indistinguishable synergistic activity. The synthetic lacticin 3147 also had no activity against the natural producing strain, indicating recognition of the synthetic peptide by the bacterial immunity factors. The chemical synthesis of lacticin 481 was more challenging due to the presence of three overlapping rings in its structure (85). In this study, analogs of lacticin 481 were produced with aberrant lanthionine LL stereochemistry instead of the normal DL stereochemistry. The DL lacticin 481 analog had similar activity to the natural lantibiotic, while the LL diastereomers had no activity. The lack of activity demonstrates the importance of the lanthionine stereochemistry for bioactivity. Chemical synthesis of epilancin 15X, produced by *S. epidermis* 15x154, was used to determine the importance of the lantibiotic's N-terminal region (86). Epilancin 15X rings BC are homologous to rings DE in nisin. The N-terminal region of epilancin 15X does not have the lipid II binding domains found in nisin's rings AB. The full length

synthetic variant of epilancin 15X had similar activity to the natural variant, but the truncated form (Δ1-8) had a hundred-fold reduction in activity against the indicator strain *S. carnosus*. The ability to synthesize even overlapping lanthionine rings shows the tremendous progress in the field of lantibiotic chemical synthesis. Chemical synthesis of lantibiotics, even with multiple complicated synthesis steps, has a total synthesis yields between 2% to 10% (82-86). Direct comparisons between natural product isolation and chemical synthesis have not been adequately addressed in the literature. Complete synthesis of lantibiotics has allowed researchers to develop new methods to produce lantibiotics containing lanthionine mimics.

I.3.2 *Synthesis of lanthionine mimics*

Oxidation of the lanthionines in nisin was shown to abolish both binding to lipid II and activity (34). This observation could prove similar to other lantibiotics, effectively limiting their application. Conversely, only one single lantibiotic has been shown to have a naturally oxidized lanthionine ring. The last ring in actagardine is composed of an oxidized lanthionine ring, which has been shown to be important for both the activity and solubility of the lantibiotic (87, 88). To prevent oxidation of the sulfur group, multiple groups have attempted to substitute the lanthionine ring with alkene, alkane, or even oxygen groups (Figure 1.3C). This was attempted in producing ring AB and ring ABC variants of nisin with either alkene or alkane bonds using ring-closing metathesis (Figure 1.3C) (89, 90). These fragments were tested for binding to lipid II and none of these variants could bind to lipid II with the same affinity as the natural fragments. Rings

DE alkene mimics were also synthesized but could not be tested fully due to the requirement of rings AB for binding to lipid II (89, 91). A ring expanded alkene variant of lacticin 3147 Ltnβ was produced by SPPS (92). This variant had no activity by itself and lacked synergy with lacticin 3147 Ltnα. Further lanthionine modifications in lacticin 3147 Ltnβ were made in which the lanthionine sulfurs were substituted with oxygens. The oxygen variant of lacticin 3147 Ltnβ had significantly lower intrinsic activity, and the variant could not work synergistically with lacticin 3147 Ltnα. In addition, a variant containing only lanthionine rings instead of methyllanthionine rings was created, referred to as (Bis(desmethyl) Ltnβ (Figure 1.3C) (93, 94). In contrast to the oxygen variant, the bis(desmethyl) variant lost intrinsic activity, but retained its synergistic activity with lacticin 3147 Ltnα . In lactocin S, the ring A lanthionine was substituted with a lanthionine mimic diaminopimelate (DAP). This substitution in lactocin S retained full activity against *P. acidilactici* Pac 1.0 (Figure 1.3C) (95). This study does demonstrate that lanthionine mimics in some systems could retain activity. However, the activity of many of the lanthionine mimics is lower compared to its natural counterpart. These observations do suggest that the lanthionine ring is important for optimal activity of lantibiotics. However, given the susceptibility of lanthionines to oxidation, methods and studies to identify lanthionine mimics to circumvent potential oxidation problems should continue to be explored.

Chemical synthesis allows researchers to bypass the limits of ribosomal synthesis of lantibiotics. The successful chemical synthesis of a variety of lantibiotics paves the way for further studies aimed at promoting the development of lantibiotics for treating

infectious diseases. Chemical synthesis provides advantages over *in vivo* production by enabling the synthesis of new structural analogs and facilitates higher yields of product when the producing strain is not capable of making sufficient quantities. Currently, the structural analogs of lantibiotics produced by SPPS are relatively small and further studies involving the incorporation of conventional and non-proteinogenic amino acids have not been adequately screened for improvements in activity. The bioactivity studies have been limited to only a few indicator strains. Additionally, the incorporation of all the unique PTMs found in lantibiotics have not been demonstrated by a SPPS approach, for instance the AviCys residue found in the epidermin group of lantibiotics have not yet been reported. Furthermore, there is still a need to optimize the synthesis of the orthogonal lanthionine rings for their integration into conventional SPPS approach for promoting a financially viable method to making lantibiotics. Semi-synthetic methods of production could combine the strengths of both chemical and *in vivo* production of lantibiotics.

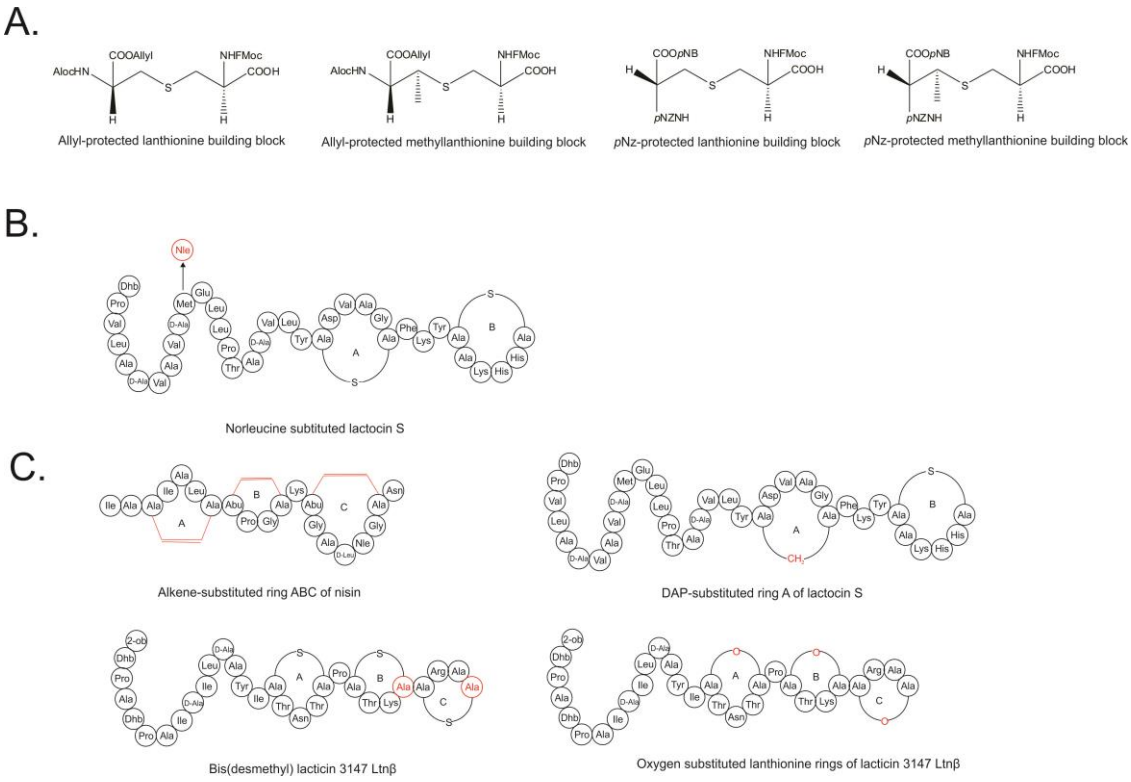

Figure 1.3 Representation of synthetic lantibiotic building blocks and fully synthesized lantibiotic variants. **A**) Chemical structures of commonly used orthogonally protected lanthionines (79-81). Abbreviations of side chains are: Fmoc: fluorenylmethyloxycarbonyl, Aloc: allyloxycarbonyl, and pNb: *p*-nitrobenzyl. **B**) Chemically synthesized variant of lactocin S with norleucine substituted at position 12 (83). **C**) Covalent structures of lantibiotics chemically synthesized with lanthionine mimics or lanthionine substitutions (89-95). The modifications are highlighted in red.

I.4. *in vitro* biosynthesis of lantibiotics

I.4.1 *in vitro mutasynthesis*

In vitro synthesis is another method wherein enhanced analogs of lantibiotics can be produced. Producing lantibiotics by an *in vitro* method bypasses several potential problems that may occur in an *in vivo* expression system. For instance, enhancement of lantibiotic activity may be toxic to the producing strain, thus, restricting these types of studies. Furthermore, some variants may not be efficiently synthesized by the producing strain and would hinder their isolation and bioactivity characterization studies. *In vitro* synthesis of lantibiotics, especially for class I lantibiotics is challenging due to using multiple enzymes for complete biosynthesis. In class II lantibiotics, the presence of the single bifunctional enzyme LanM makes *in vitro* synthesis less complicated (Figure 1.1). Furthermore, studies into the kinetics of various LanM enzymes could lead to the successful application of LanM synthetases to other peptides (96, 97).The successful *in vitro* synthesis of a lantibiotic has led to the development of an *in vitro* technique referred to as mutasynthesis, wherein chemically or ribosomally synthesized precursor peptides are modified by *in vitro* reconstituted lantibiotic synthetases. The production of enhanced analogs of lacticin 481 has been accomplished using *in vitro* mutasynthesis. Analogs of lacticin 481 were created by first making lacticin precursor peptides by SPPS containing non-proteinogenic amino acids, the PTM containing lanthionine rings were subsequently introduced *in vitro* by using the bifunctional enzyme LacM (43, 98). Of the 11 analogs containing non-proteinogenic amino acids, two were found to have enhanced antibacterial activity. The substitution of either a homophenylalanine or a napthylalanine

resulted in a significant increase in activity. In another lantibiotic system, *in vitro* reconstitution of both HalM1 and HalM2 enzymes for the two component lantibiotic haloduracin A1 and haloduracin A2 allowed for structural and mode of action studies to be performed on these peptides (99, 100). Although promising, *in vitro* mutasynthesis has been limited to class II lantibiotics. Given current developments in reconstructing *in vitro* activities of class I synthetase LanB, the synthesis of class I lantibiotics by in *vitro* mutasynthesis will be accomplished soon (101). Both the structures of NisC and NisB have been elucidated, which could potentially lead to the *in vitro* synthesis of class I lantibiotics (102, 103). The synthesis of biologically active nisin using bacterial extracts has been previously shown, thus, the use of purified PTM enzymes should be possible (104).

I.4.2 *Lantibiotic Coupling (Semi-synthetic)*

Coupling of lantibiotics provides another opportunity to make novel peptide variants enabling new applications and functions. The potential applications of this approach include the development of target specific bacterial probes, expanding the bioactivity of the lantibiotic, and provide a protective layer by coating lantibiotics on medical equipment (105, 106). The conjugation of lantibiotics with various compounds has been the subject of several studies. For instance, conjugating nisin with select functional groups has the potential for producing more stable and soluble analogs. PEGylation of nisin was attempted, but this approach led to inactive analogs due to the acylation of the lysine residues on nisin (105). Conversely, biotinylation of the C-

terminus of nisin by EDC coupling resulted in a functional analog that retained activity against *Kocuria rhizophilia* (107). N-terminally thiolated nisin variants with poly(ethylene oxide)/poly(propylene oxide)/poly(ethylene oxide) triblocks have been synthesized (108). These coupled variants of nisin exhibited similar activity to wild-type nisin. Since the nisin AB rings are important for lipid II binding, this truncated form of nisin has been utilized for forming antibiotic hybrids that may promote dual functionality for binding antimicrobial targets. Nisin AB (1-12) was conjugated to vancomycin using click chemistry (109). Since both antibiotics targeted lipid II, albeit at different regions of lipid II, it was hypothesized that binding nisin to vancomycin would increase the variants affinity for lipid II and ultimately increase activity against vancomycin resistant strains. The nisin (1-12)–vancomycin hybrid was shown to restore activity against vancomycin resistant enterococci than vancomycin (109). Previously, as described above, an alkene substituted ring DE fragment of nisin could not be tested due to the requirements of rings AB for binding to lipid II. Using the same click chemistry method, the alkene rings DE were fused to nisin's rings ABC to determine the effects of the alkene substitution on these rings (110). Unfortunately, the DE nisin hybrid variant did not exhibit pore formation activity nor did it promote binding to lipid II. To help facilitate conjugation of nisin at the C-terminus, a variant containing a C-terminal alkyne was developed to promote conjugation of various azide containing molecules by click chemistry. Initial conjugation with fluorophores yielded nisin conjugates that retained activity (106). In an attempt to potentially expand the spectrum of activity of lantibiotics to Gram-negative bacteria, gallidermin lysine residues were conjugated with various

siderophores. It was believed that the conjugated siderophores would facilitate the transport of gallidermin across the outer membrane. Although, the conjugates retained activity against the Gram-positive indicator strain *L. lactis* subsp. cremoris HP, the conjugated variants did not yield any activity against Gram-negative bacteria (111). NVB302 is a semi-synthetic variant of deoxyactagardine with a synthetically introduced C-terminal 1, 7 diaminoheptane tail (112). This variant has been shown to have greater solubility and activity compared to deoxyactargardine. NVB302 is being developed for the treatment of *C. difficile and* is currently in a phase I clinical trial, successfully showing a semisynthetic variant of an existing lantibiotic can be developed for further uses (113).

In vitro mutasynthesis and lantibiotic conjugation offer unique opportunities to engineer enhanced variants of lantibiotics or facilitate new lantibiotic applications. *In vitro* mutasynthesis combines both the strengths of chemical synthesis with the ease and practicability of using the PTM enzymes. Unfortunately, *in vitro* mutasynthesis is currently limited to producing class II lantibiotics. The conjugation experiments of lantibiotics could potentially broaden the scope of lantibiotic applications. However, conjugation experiments have generally led to a decrease in lantibiotic activity. The conjugate may be interfering with lantibiotic's ability to access or bind to its target. The chemical substitutions may perturb important functional elements within the lantibiotic. The combination of conjugation methods and SPPS by conjugating SPPS building blocks may enable the creation of larger structural libraries that can be screened for

improvements in activity. For now, the challenge of lantibiotic conjugation is a balance between retaining activity and introducing a new or enhanced function.

I.5. Conclusions

The three different methods: (i) *In vivo* engineering of variants through amino substitutions, (ii) incorporation of proteinogenic and non-proteinogenic amino acids or lanthionine mimics through complete chemical synthesis or *in vitro* mutasynthesis, and (iii) chemical coupling of lantibiotics are useful in the development of novel lantibiotics. These techniques have furthered our understanding of structurally important regions for activity and have also led to the development of lantibiotic variants with an increase in inhibitory activity against medically relevant bacterial pathogens. The multipronged approaches for engineering novel lantibiotics have expanded our understanding of lantibiotic function and have provided evidence that they are all useful methods for improving the spectrum of activity of current lantibiotics.

I.6. Future directions

Lantibiotics are active against a broad spectrum of Gram-positive bacteria, including multi-drug resistant pathogens. Several different approaches for producing novel lantibiotic analogs have demonstrated their potential for developing lantibiotics with improved *in vitro* inhibitory activities against pathogenic bacteria. However, some of the reports on the bioactivity of novel lantibiotic analogs were limited. For instance, several studies performed on nisin only tested the activity against a few strains of

pathogenic and non-pathogenic bacteria (53-55, 57). Rescreening the activity of these structural variants against a full spectrum of medically relevant Gram-positive bacteria may elucidate the analog's medical application. A more in-depth understanding of the variation in the activity of these analogs against a larger spectrum of bacteria may promote site-specific engineering studies that can further improve their activity against specific bacterial species. In addition, some of the analogs that were considered to have a reduction in activity may actually have superior activity against bacteria in a larger screen. The reasoning for the increase in activity of these structural analogs against different bacteria is not understood. Additional studies aimed at understanding whether the modification promotes binding to the microbial target need to be done. For instance, lipid II is the biological target for many of the class I and class II lantibiotics. If some of the structural modifications improve lipid II affinity or access to lipid II, this information may lead to an improved approach for producing enhanced analogs.

The efforts up to now have been an erudite exercise demonstrating that lantibiotics can be synthesized with activities superior to the native compounds. These approaches have not yet been fully applied toward the development of a new lead compound for clinical development. Many factors need to be taken into consideration for the development of a lead compound. The half-life and distribution of an antimicrobial compound is just a couple of important factors that need to be addressed. The half-life of lantibiotics like nisin and mutacin 1140 are relatively short (14, 36). To date, there have not been any reports showing improvements in the pharmacokinetic activities of lantibiotics. These studies will be more significant than the reported improvements in the

inhibitory activity against Gram-positive pathogens, given that lantibiotics already have submicromolar and nanomolar activities. The *in vivo* and synthetic methods aimed at expanding lantibiotic function were all done on relatively small analog libraries. With this realization, significant efforts have hardly even begun until studies begin to explore the full breadth of proteinogenic and non-proteinogenic amino acid substitutions. These studies should not only take into consideration microbial inhibitory activity, but also activities that will improve the pharmacokinetic and pharmacodynamic activities of the native compound.

Commercially viable methods for large scale synthesis are a limiting factor for several lantibiotics and are a major problem that has hampered the development of lantibiotics for therapeutic use. Even though some of the *in vivo* synthesized analogs did significantly increase lantibiotic production, *E. coli* expression or chemical synthesis may be the best method to produce lantibiotics in large scale. *In vivo* engineering can be cumbersome due to numerous issues, such as the lack of genetic tools and the lack of growth conditions for producing the lantibiotic. The movement of the biosynthesis genes to a heterologous expression host could circumvent these problems. In the review, we did mention the expression of lichenicidin in *E. coli* and the expression of mersacidin in a genetically competent bacilli species. The movement of other lantibiotic biosynthesis genes into a heterologous expression host has been accomplished and using this approach for other lantibiotic systems may improve their production and bioactivity characterization studies. However, *E. coli* expression systems for lantibiotics are limited to the complexity of the biosynthesis pathway for the lantibiotic and have limitations in

lantibiotic transport. A commercially viable production of lantibiotics using an *E. coli* system has not yet been reported. Recently, Süssmuth et al have been able to isolate lichenicidin from *E. coli*, but yields were still low enough to be considered nonviable (114). *In vitro* mutasynthesis is still relegated to class II lantibiotics, but has the potential to synthesize class I lantibiotics. Furthermore, *in vitro* mutasynthesis can be used to modify other non-lantibiotic peptides, which may also facilitate the production of unique peptides with nonantimicrobial applications (115-117). The relative stability or robustness of the PTM enzymes, as well as the yield of fully modified peptides from the *in vitro* mutasynthesis approach have not been adequately reported. Although promising, chemical synthesis of lantibiotics requires a profusion of protection and de-protection steps. Improvements in these chemical steps may facilitate commercially viable yields of lantibiotics.

Advances in next generation sequencing and techniques for cultivating hard to grow bacteria will facilitate the discovery of new lantibiotics. Some of these lantibiotics may reveal novel PTM modifications and structures. Although a vast majority will probably be related to currently known lantibiotics, the differences in amino acids compositions within these naturally occurring analogs will point toward regions that are amenable to modification. The knowledge of these naturally variable regions will assist in facilitating studies at promoting lantibiotic function. Multiple methods of producing novel lantibiotic analogs have increased the scope of our understanding of lantibiotic structure and function. These methods have also promoted the potential for lantibiotics to augment our current repertoire of clinically used antibiotics.

CHAPTER II:

THE LEADER PEPTIDE OF MUTACIN 1140 HAS DISTINCT STRUCTURAL COMPONENTS COMPARED TO RELATED CLASS I LANTIBIOTICS[*]

II.1. Synopsis

Lantibiotics are ribosomally synthesized peptide antibiotics composed of an N-terminal leader peptide that promotes the core peptide's interaction with the PTM enzymes. Following PTMs, mutacin 1140 is transported out of the cell and the leader peptide is cleaved to yield the antibacterial peptide. Mutacin 1140 leader peptide is structurally unique compared to other class I lantibiotic leader peptides. Herein, we further our understanding of the structural differences of mutacin 1140 leader peptide to other class I leader peptides. We have determined that the length of the leader peptide is important for the biosynthesis of mutacin 1140. We have also determined that mutacin 1140 leader peptide contains a novel four amino acid motif compared to related lantibiotics. PTM enzyme recognition of the leader peptide appears to be evolutionarily distinct from related class I lantibiotics. Our study on mutacin 1140 leader peptide provides a basis for future studies aimed at understanding its interaction with the PTM enzymes.

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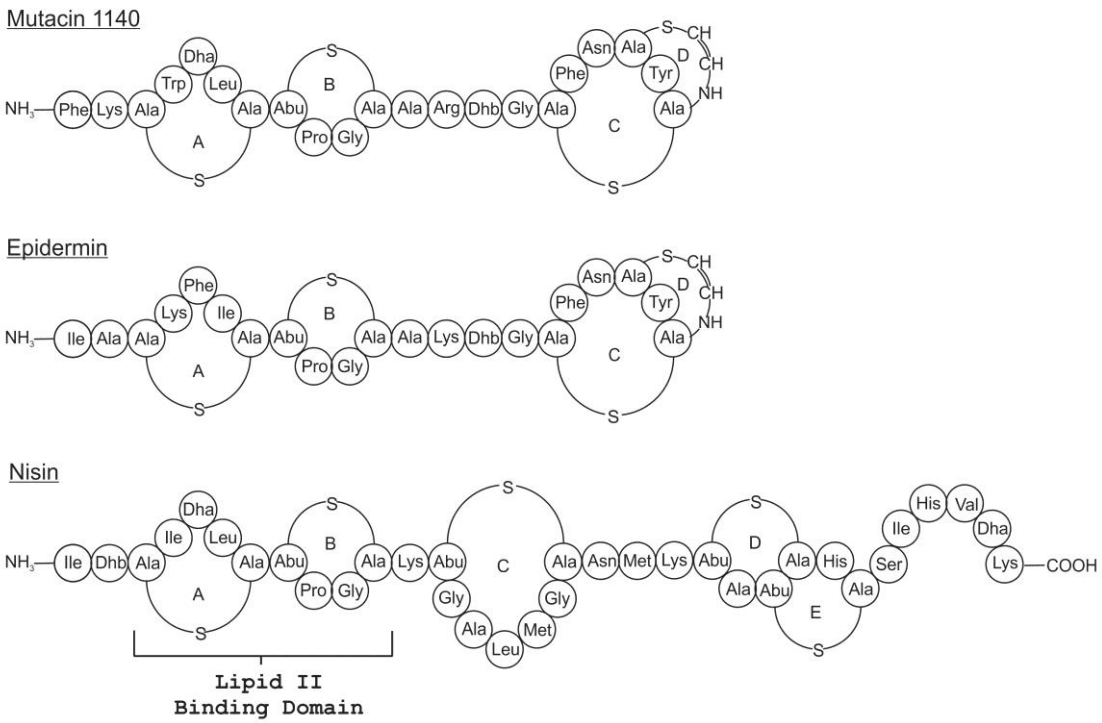
[*] Reprinted with permissions from The leader peptide of mutacin 1140 has distinct structural components compared to related class I lantibiotics by Jerome Escano, Byron Stauffer, Jacob Brennan, Monica Bullock, and James Smith. 2014. *MicrobiologyOpen*. 3:6. Copyright [2014] by Escano et al 2014 DOI: 10.1002/mbo3.222

II.2. Introduction

Many strains of medically important bacteria have become increasingly resistant to currently available antibiotics. Healthcare associated infections caused by multi-drug resistant pathogens are leading to longer hospital stays and increased mortality. Worldwide, millions suffer from antibiotic-resistant infections, which results in a huge cost to the healthcare system. The development of new antibiotics has become a critical, unmet need in the medical community (118-120). Lantibiotics are an important class of antibiotics with potential clinical relevance for the treatment of antibiotic resistant Gram-positive bacteria (81, 121-123). Lantibiotics acquired their name because of the characteristic lanthionine rings found in the bioactive core peptide. Lantibiotics also contain an array of unusual amino acids such as Dha, Dhb, AviCys, aminobutyrate (Abu), 2-oxopropionyl, 2-oxobutyryl, and hydroxypropionyl (81, 121-123). The molecular structure of mutacin 1140 contains four macrocyclic rings (see Figure 2.1A), each of which contains a lanthionine or methyllanthionine residue. Mutacin 1140 also contains the posttranslational modified amino acid residues Dha, Dhb, and AviCys (29). Mutacin 1140 rings A and B (Figure 1A), the lipid II binding domain, is similar to the class I lantibiotics nisin and epidermin (24). It was discovered that both nisin and mutacin 1140 abduct lipid II from the site of new cell wall synthesis, ultimately causing cell death (51, 52).

A



B

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                                                                  -1
Mutacin 1140      MSNTQLLEVLGTETFDVQEDLFAFDTTDTTIVA-SNDDPDTR
Nisin                   MSTKDFNLDLVSVSKK--DSGASPR
Subtilin                MSKFDDFDLDVVKVSKQ--DSKITPQ
Epidermin            MEAVKEKNDLFNLDVKVNAKESNDSGAEPR
Gallidermin          MEAVKEKNELFDLDVKVNAKESNDSGAEPR
Pep5                    MKNNKNLFDLEIKKETSQNTD-ELEPQ
Epilancin K7             MNNSLFDLNLNKGVETQKSDSLSPQ
```

C

Figure 2.1 Lantibiotic structural elements. **A)** Covalent structures for mutacin 1140, epidermin, and nisin with the lanthionine rings labeled from N- to C-terminus. **B**) Leader sequence alignments of structurally related class I type AI lantibiotics; i.e. mutacin 1140 produced by *S. mutans*, nisin produced by *L. lactis*, subtilin produced by *B. subtilis*, epidermin produced by *S. epidermidis*, gallidermin produced by *S. gallinarium*, Pep5 produced by *S. epidermidis*, epilancin K7 produced by *S. epidermidis* (23, 124, 145-150). **C**) Secondary structure prediction using SOPMA for mutacin 1140 leader peptide; h (alpha helix), e (extended strand), c (random coil), t (beta turn). Alpha helical regions are in bold, while random coils are underlined in the leader peptide sequence.

Mutacin 1140 is synthesized by the Gram-positive oral bacterium *Streptococcus mutans* JH1140 (124). Mutacin 1140 was shown to have low micromolar to submicromolar minimum inhibitory concentration (MIC) against several species of Gram-positive pathogens (10). The study by Ghobrial et al. further demonstrated that mutacin 1140 is bactericidal against *Streptococcus pneumoniae* and multi-drug resistant strains of *Staphylococcus aureus*, bacteriostatic against vancomycin-resistant *Enterococcus faecium* (VREF), and had no activity against Gram-negative bacteria or yeast. The study demonstrated that mutacin 1140's time-kill profile against select pathogens was similar to those of vancomycin, which also binds to lipid II (125). Furthermore, mutacin 1140 had a low *in vitro* cytotoxicity, was well tolerated in murine models when administered intravenously, and was found to be distributed in all body compartments. Demonstration of efficacy was achieved in a pilot study in which 60 times the LD_{50} of *Staphylococcus aureus* was administered in a rat peritonitis model (14). Development of significant resistance was not observed during repeated subculture of *S. aureus* or *S. pneumoniae* in medium containing sub-lethal concentrations of mutacin 1140 (10). The basis for this observation may be due, in part, to the fact that the molecular target, the isoprene, pyrophosphate, and N-acetylmuramic acid of lipid II, is evolutionarily ancient and highly conserved throughout the bacterial kingdom, indicating that mutations within this structural element of lipid II may be prohibited. Based on these and other studies, mutacin 1140 has the potential to replace current, failing drugs of last resort and serve in the treatment of problematic infections caused by Gram-

positive bacteria such as methicillin resistant *S. aureus*, vancomycin resistant Enterococci, and *Clostridium difficile*.

A better understanding of the biosynthesis of mutacin 1140 may promote the production and purification of mutacin 1140 or core peptide variants of mutacin 1140. Several core peptide variants of mutacin 1140 have been previously engineered to have enhanced bioactivity (40). The mutacin 1140 leader peptide sequence is distinct from the leader peptide of related class I lantibiotics (Figure 2.1B). Class I leader peptides have conserved structural elements. The common motif found in class I lantibiotics is the F(N/D)LD box and a proline at the (-2) position. Several mutagenesis studies with the lantibiotic nisin biosynthesis system show that the box motif is important for the maturation of the core peptide antibiotic (126-128). Additional experimental studies suggest that the leader peptide is important for interacting with the dehydratase LanB, cyclase LanC, and transporter LanT (117, 129, 130). However, these studies are challenged by the failure to distinguish between which of the proposed leader peptide functions are affected by the changes in amino acid residues.

In this study, we have identified structural regions in the leader peptide that are important for the biosynthesis of mutacin 1140 core peptide. We have determined that the leader peptide function is not dependent on the amino acid sequence except for a four amino acid region within the center of the leader peptide. These four amino acids comprise a novel four amino acid motif with the latter two amino acids being essential for leader peptide function. This motif is different in amino acid properties found in other class I lantibiotic motifs in related lantibiotic groups such as nisin and epidermin.

Mutacin 1140 leader peptide is also longer than other class I lantibiotic leader peptides and we have determined that the additional length of the peptide of mutacin 1140 is important for efficient biosynthesis. These studies advance our understanding of the biosynthesis of mutacin 1140 and will promote future studies aimed at furthering our understanding of the leader peptide's interaction with the PTM modification enzymes.

II.3. Results

II.3.1 *Length of the leader peptide affects biosynthesis*

The mutacin 1140 leader peptide (Figure 2.1B) is 18 and 11 amino acids longer than nisin A and epidermin leader peptides, respectively. Small consecutive truncations of four and five amino acids were made starting at the (-40) position corresponding to the amino acid after N-terminal methionine (Figure 2.2A). Deletion mutations between residues Δ(-40 to -37), Δ(-36 to -33), Δ(-32 to -28) were investigated to determine whether there are regions of structural importance in the extended leader peptide (Figure 2A). These mutations had little impact on the bioactivity of the mutant strains as determined by deferred antagonism assays (Figure 2.2B). This assay is a sensitive quantitative measurement of bioactivity for mutacin 1140 production (40). Each strain of *S. mutans* is grown under identical conditions and the bioactivity is assessed by calculating the percent differences in the area of the zone of inhibition between mutant strains and wild-type strain. Reductions in activity suggest that less of mutacin 1140 is made or the biosynthesis of mutacin 1140 by the bacterium is altered leading to the synthesis of less active products. Progressively longer truncations from residues Δ(-40 to

-33) and Δ(-40 to -28) were subsequently measured for bioactivity (Figure 2.2A and 2.2B). The deletion mutants were reduced in bioactivity and the loss in bioactivity increased with the length of the deletion. The progressive loss in bioactivity with increasing size of deletion suggests that the length of the leader peptide is important for the biosynthesis of mutacin 1140. Mutacin 1140 was isolated from the Δ(-40 to -33) deletion mutant and its mass was 2265 Da, as predicted for a core peptide that has successfully undergone all dehydrations and decarboxylation (Table 2.1). There was no cyanylation of free thiols by CDAP, suggesting that all lanthionine rings were formed by the cyclase (Figure 2.3). The substitution of six residues (-40 to -35) with histidines resulted in a statistically significant decrease in bioactivity, whereas, an insertion of six histidines between residues (-41 and -40) position resulted in no significant loss in bioactivity (Figure 2B). These results further emphasize the importance of the leader peptide's predicted secondary structure for bioactivity (Figure 2.1C). Secondary structure analysis using SOPMA (131) predicts that the N-terminal end of the leader peptide is an alpha helix, while the C-terminal end is a random coil (Figure 2.1C). The insertion of six histidines at the N-terminal end did not affect bioactivity because they presumably extend outside of a binding cleft, whereas the substitution of six histidines is within the leader peptide binding cleft of a PTM enzyme. The substitution of six histidines within this region is predicted by SOPMA to change the secondary structure to a random coil. Furthermore, the substitution of six histidines within this region would contribute to steric interference of binding. These results suggest that length of the first half of the leader peptide sequence, and possibly secondary structure, is more important for the

biosynthesis of mutacin 1140 than the actual amino acid sequence. The lack of sequence specificity for the leader peptide has also been reported in other lantibiotic systems (127, 128, 132, 133).

Table 2.1 MALDI-MS data for isolated mutacin 1140 products from *S. mutans* JH1140 leader peptide mutants.

Strain	Mass (Da)
S. mutans ATCC 55676	2264±1
S. mutans Δ(-40-33)	2264±1
S. mutans Δ(-40-28)	N.D.
S. mutans Δ(-40-24)	N.D.
S. mutans Δ(-40-37)	2264±1
S. mutans Δ(-36-33)	2264±1
S. mutans Δ(-32-28)	2264±1
S. mutans Δ(-27-24)	2264±1
S. mutans Δ(-23-20)	N.D.
S. mutans F(-27)A	2264±1
S. mutans D(-26)A	2264±1
S. mutans V(-25)A	2264±1
S. mutans Q(-24)A	2264±1
S. mutans Q(-24)insA	2264±1
S. mutans E(-23)A	2264±1
S. mutans E(-23)insA	2264±1
S. mutans D(-22)A	2264±1
S. mutans D(-22)insA	N.D.
S. mutans L(-21)A	2264±1
S. mutans Δ(-21)	2264±1
S. mutans L(-21)insA	2264±1
S. mutans F(-20)A	2264±1
S. mutans Δ(-20)	2264±1
S. mutans Δ(-19)	2264±1
S. mutans AALF	2264±1
S. mutans EDED	N.D.
S. mutans N+6xhis	2264±1
S. mutans N6xhis	2264±1

II.3.2 *Mutations in the presumed FNLD box did not change activity*

A comparison of leader peptide sequences of class I lantibiotics shows a conserved sequence within the first half of the leader peptide (Figure 2.1B). The F(N/D)LD box appears to be highly conserved among different lantibiotics (123, 128), but the predicted box sequence for mutacin 1140 is different compared to other lantibiotics. The box for mutacin 1140 was predicted to have the sequence FDVQ located at the (-27 to -24) region of the leader peptide (121). We individually substituted the amino acids FDVQ with alanine to determine their importance for bioactivity (Figure 2A and 2C). Alanine substitutions did not exhibit any change in activity. Given the possibility that the effect of single point mutations could be masked by the presence of other amino acids within the sequence, we deleted the whole FDVQ box Δ(-27 to -24) from the leader peptide. The truncation also did not significantly reduce the bioactivity (Figure 2.2A and 2.2C). We isolated the lantibiotic from the culture and determined the mass. The mass of the mutant product in combination with its bioactivity suggested that the core peptide underwent all posttranslational modifications (Table 2.1). These observations are incompatible with what has been reported for other related lantibiotics. The substitution at the F(-18) position of nisin with an alanine was shown to reduce the bioactivity of this strain and the deletion of the F(N/D)LD box of nisin abolished production of the lantibiotic (127). We therefore searched downstream for a new box.

II.3.3 *A novel four amino acid motif affects biosynthesis*

Considering how the deletion of the predicted motif for mutacin 1140 did not affect production, additional deletion mutants were made to determine whether or not mutacin 1140 leader peptide has a unique "box motif" as has been characterized for nisin (126, 127, 134, 135). A deletion of four residues Δ(-23 to -20), positioned at the C-terminal end of the predicted FDVQ box described above, resulted in a complete loss in bioactivity (Figure 2.2A and 2.2D). The box has an "EDLF" motif, which is a different primary sequence from other related lantibiotics. The loss of activity may be attributed to a loss in leader peptide affinity to the binding cleft of a PTM enzyme (126, 127, 134, 135). However, the deletion of seventeen amino acids Δ(-40 to -24) resulted in nearly a complete loss in bioactivity, suggesting that this region is also capable of stabilizing an interaction within the binding cleft of a PTM enzyme. It would be unusual for the loss of just four amino acids Δ(-23 to -20) to result in a complete loss of activity, unless this region is important for anchoring the leader peptide at the correct position in the binding cleft or is essential for activating a functional conformation of one of the PTM enzymes. To test the importance of site specific amino acids that are necessary for binding, we substituted the EDLF box with alanine residues. Each of these alanine substitutions for E(-23)A, D(-22)A, and L(-21)A resulted in no loss in bioactivity (Figure 2.2A and 2.2D). The alanine substitution for F(-20)A resulted in approximately a 24% reduction in bioactivity. An insertion of alanine residues in the region of the putative EDLF box was evaluated. The Q(-24)InsAla and E(-23)InsAla mutants did not disrupt the bioactivity. The D(-22)InsAla mutant resulted in an 88% reduction in activity, while the L(-

21)InsAla did not result in any significant loss in bioactivity. Furthermore, a deletion at A(-19) position did not result in any reduction in bioactivity. Yet, a deletion at L(-21) or F(-20) resulted in approximately 25% reduction in activity. These mutations point to the importance of all four of the EDLF amino acids in the box. For example, EDLF, EADLF, EDLAF, DLF, and EDF still have normal bioactivity, while EDLA, EDF, and EDALF had a significant reduction in bioactivity (Figure 2.2A and 2.2D). The relative position of the acidic amino acids (ED) from the hydrophobic amino acids (LF) appears to be important for bioactivity. Each of the acidic and hydrophobic amino acids is able to compensate for the substitution of a single alanine residue in the sequence without resulting in a large loss in bioactivity. To test the requirement of an acidic amino acid relative to the position of hydrophobic amino acid, the mutants AALF and EDED boxes were tested. Each of these mutations resulted in a significant loss in bioactivity (Figure 2.2A and 2.2D). AALF activity was reduced by ~25% and EDED activity was reduced by ~65%. These mutations further indicate the importance of the EDLF box, specifically the latter two amino acids in the motif. This motif could be a potential binding site for the dehydratase LanB. We were unable to isolate any product from the culture liquor in the EDED mutant strain, which suggests that the motif is important for biosynthesis. We were able to isolate the AALF mutant product which had a mass of 2265 Da. Furthermore, all of the substitution or insertion mutations within this region resulted in a product with a mass of 2265 Da, which is the mass of the product that has undergone all dehydration and decarboxylation modifications (Table 2.1). There was no cyanylation of free thiols by CDAP in the AALF, ΔL-20, ΔF-19, and F-19A box mutants, suggesting

that all lanthionine rings were formed by the cyclase (Figure 2.3). The reduction in bioactivity in connection with the isolation of only fully modified mutacin 1140 supports the notion that core peptide PTM enzyme modifications are important for transport.

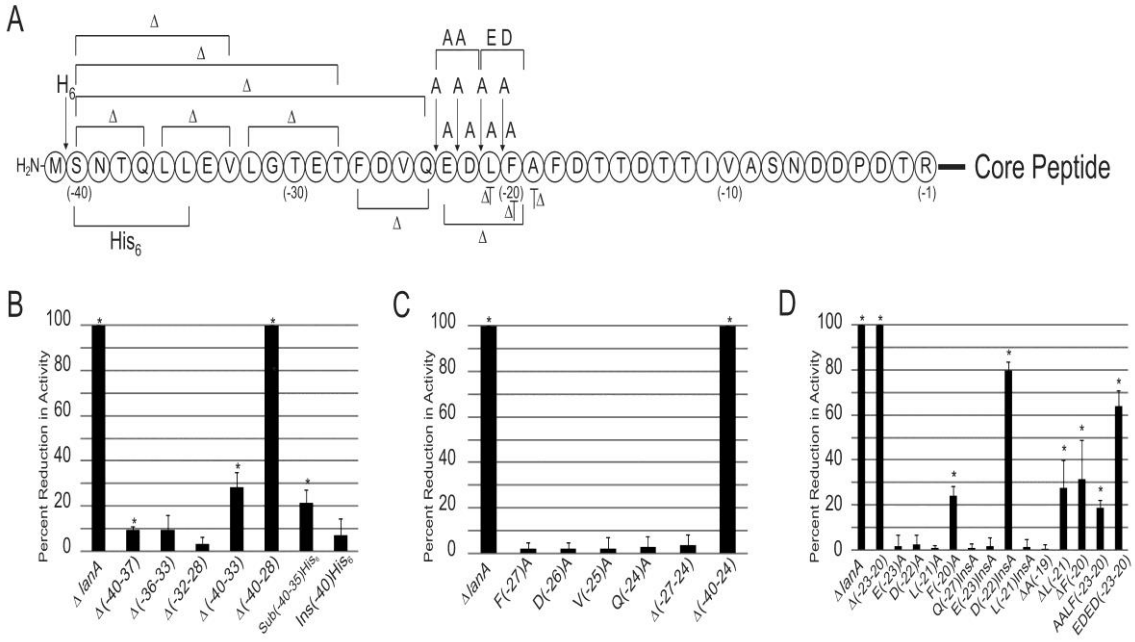

Figure 2.2 Identification of structural elements within the mutacin 1140 leader peptide that are important for bioactivity. **A**) Covalent structure representation of the mutations made on the leader peptide. Bioactivity for leader peptide mutants were measured as the percent difference in the zone of inhibition between wild-type and the mutant strains. Δ*lanA* strain was used as a negative control for bioactivity in all experiments. The change in activity was measured for: **B**) N-terminal deletions of the leader peptide, **C**) mutations in the proposed FNLD-type box, **D**) mutation in a new box, For each mutation, the bioactivity has been compared to the activity of wild-type *S. mutans* JH1140 strain. Statistical method used was Student t-test and the asterisk signifies statistical significance ($p<0.05$).

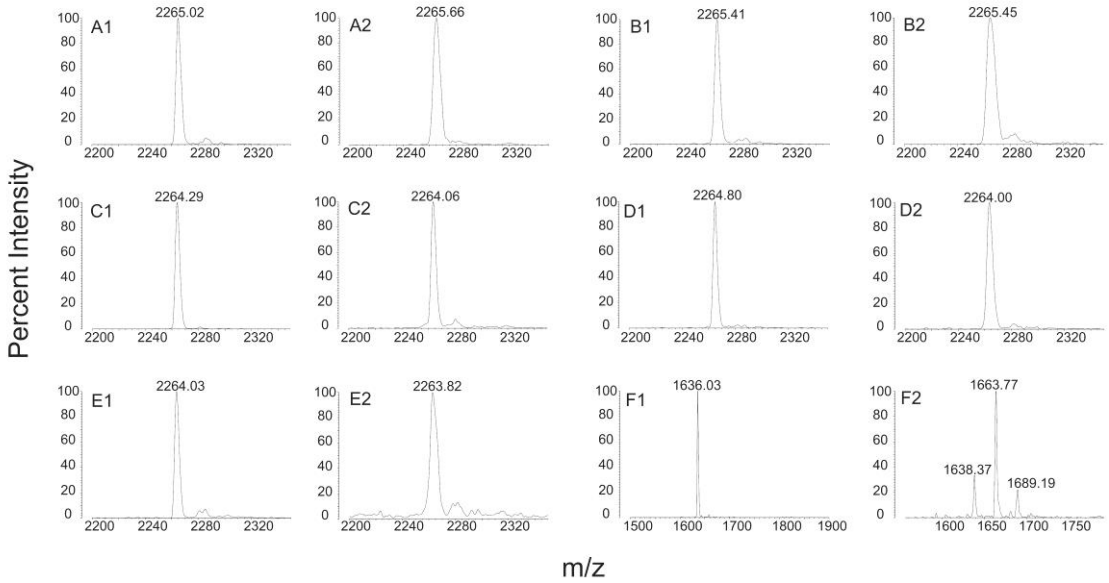

Figure 2.3 Cyanylation of free thiols by CDAP. **A**) MALDI-MS of peptide from Δ(-40-33) strain (A1) and CDAP treated peptide (A2); **B**) MALDI-MS of peptide from AALF strain (B1) and CDAP treated peptide (B2); **C**) MALDI-MS of peptide from ΔL-20 strain (C1) and CDAP treated peptide (C2); **D**) MALDI-MS of peptide from ΔF-19 (D1) and CDAP treated peptide (D2); **E**) MALDI-MS of peptide from F-19A (E1) and CDAP treated peptide (E2); **F**) MALDI-MS of positive control peptide resact (F1) and CDAP treated peptide (F2). None of the isolated peptides from *S. mutans* mutant strains reacted with CDAP, while the positive control was cyanylated.

II.4. Discussion

Due to their unique structure, lack of resistance, and wide array of activity, lantibiotics have become a prime candidate for development of new therapeutics. Spontaneous resistance to lantibiotics like mutacin 1140 is highly unlikely given their mechanism of action (50-52, 136). Mutacin 1140, a lantibiotic produced by *S. mutans* JH1140, has been shown to be active against serious pathogens like MRSA (10). In this study, we investigated the role of structural regions within the leader peptide and core peptide for biosynthesis. Mutacin 1140 leader peptide sequence is different and longer than other lantibiotic leader peptides, while the core peptide sequence is similar to epidermin and nisin (Figure 1). A better understanding of the role of the leader peptide in the posttranslational modification of lantibiotics will allow us to advance the use of the PTM enzymes for the synthesis of mutacin 1140, novel lantibiotics, and therapeutic peptides.

In nisin, N-terminal mutagenesis studies upstream of the conserved FNLD box were tolerated by the PTM enzymes (137). We predicted that N-terminal truncations of the mutacin 1140 leader peptide would also be well tolerated and that the additional length may not be important for bioactivity. However, we did see a loss in activity with the increasing length of the deletion upstream of the newly identified box. Given that the small truncations of four and five amino acids over the same regions as covered by the larger deletion mutants did not reduce the bioactivity, site specific amino acids within this region of the leader peptide may not be important for recognition by the PTM enzymes. Presumably, hydrogen bonds are formed between the peptide backbone of the

N-terminal portion of the leader peptide and PTM enzyme. This type of interaction and not the interaction of the amino acid side chains of the leader peptide would account for the lack of sequence specificity. Other studies support a helical structure for the leader peptide in the binding cleft of the dehydratase of lacticin 481 and that secondary structure is important for activity (123, 138). These studies have shown that insertion of prolines, which would disrupt the helical structure, would reduce the bioactivity (123, 138). Secondary structure prediction suggests that the N-terminal portion of mutacin 1140 leader peptide is helical, while the C-terminal end is a random coil. The leader peptide sequence within the N-terminal region is presumably reserved to a subset of amino acids that will not disrupt the secondary structure or is reserved to amino acids that will not contribute to the steric hindrance of binding. Plat et al. have also reported a lack of sequence specificity within the nisin leader peptide (127).

Some models for the binding cleft suggest that both LanB and LanC have their own distinct regions for binding the leader peptide or that the interaction of LanB and LanC form the binding cleft for the leader peptide (128). There are two publications pertaining to the interaction of LanB and LanC that appear to have conflicting results. Mavaro et al. showed a 1:1 stoichiometry of binding of prenisin, dehydrated nisin, and fully modified nisin to LanB. The affinity of the prenisin with leader peptide was approximately 1 μM and the affinity of fully modified nisin with leader peptide was a log higher. The dehydrated form had a two-fold higher affinity to LanB than prenisin. This data would suggest that prenisin would prefer to associate with LanB and that cyclization may occur while being bound to LanB. Abts et al. reported the binding of

leader peptide to the cyclase LanC (126). The prenisin, dehydrated and fully modified nisin had similar binding affinities at approximately 2 μM. In this study, they showed that AALD and FNAA box mutations resulted in a complete loss of binding to LanC. However, synthesis of fully modified nisin was shown by Plat et al., while having similar mutations (127). This suggests that cyclase activity is occurring while leader peptide is bound to LanB and that LanC binding is not essential for cyclase activity. One would expect the formation of only a dehydrated nisin and not a biologically active peptide, if leader peptide binding to LanC was required. The study by Abts et al. does clearly show the requirement of the FNLD box for binding to LanC and seems to conflict with the study by Plat et al. (126, 127). In view of these results, it is possible that there are two functional leader peptide binding sites that are not competing for the leader peptide. Both sites may coordinate the binding of the leader peptide, so that the core peptide can access the catalytic sites of the PTM enzymes. Additional studies will need to be done to discern the importance of these two sites in nisin biosynthesis. Biologically active product was observed in the mutacin 1140 biosynthesis system in the EDLF box mutants AALF and EDED. The AALF mutant had a single product with the mass of a fully modified core peptide, while the peptide in the EDED mutant could not be isolated for mass characterization. The lack of CDAP derivatized products support complete lanthionine ring formation in the AALF mutant product. Mutations within mutacin 1140 leader peptide box motif have similar characteristics to the mutants reported in the FNDL box in the nisin biosynthesis system.

To date, the biosynthesis of only a handful of lantibiotics has been studied. Additional studies within other lantibiotic biosynthesis systems will promote our understanding of this dynamic process. One important goal to lantibiotic research is the application of the PTM enzymes toward the synthesis of novel therapeutic compounds. Surely, an expanded understanding of PTM enzymes in other lantibiotic systems will promote this endeavor. We believe that the EDLF box is a structurally important region that facilitates alignment of the leader peptide within the binding cleft of the dehydratase LanB, similar to the function of the FNLD box of nisin (121-123). However, the difference in leader peptide box motif and length suggest that the leader peptide binding site is evolutionarily unique to other class I lantibiotics. This brings to question, whether lantibiotics should be classified by structural elements within the leader peptide as has been previously reported (123) or should they be classified based on core peptide structure. The classification of lantibiotics by leader peptide does suggest evolutionary relatedness in their PTM systems and substrate specificities. Given the complexity of lanthipeptide biosynthesis, classification by leader peptide appears to be a more logical approach. Additional studies aimed at understanding substrate specificity of the PTM enzymes between lantibiotic systems may promote a better understanding of the evolutionary differences between the class I PTM enzymes. Our leader peptide mutagenesis study and the concomitant isolation of the mutagenic products provide a better understanding of mutacin 1140 leader peptide. Furthermore, the studies show that the leader peptide of mutacin 1140 has evolved novel structural elements that promote its interaction with its PTM enzymes and that these structural elements are unique when

compared to related class I lantibiotics. Further studies within mutacin 1140 biosynthetic pathway are underway to determine substrate specificity of mutacin 1140 biosynthesis system.

II.5. Materials and methods

II.5.1 *Bacterial strains, plasmids and media*

Bacterial strains and plasmids used in this study are listed in Table 2.2. The cloning strain *Escherichia coli* DH5α (Invitrogen, Carlsbad, CA) was cultured at 37°C on Luria-Bertani (LB) broth or agar. THyex broth (30g/L Todd Hewitt Broth, 3g/L yeast extract), THyex agar media (30g/L Todd Hewitt Broth, 3g/L yeast extract, 15g/L agar; Bacto, Sparks, MD) and Top agar media (30g/L Todd Hewitt Broth, 3g/L yeast extract, 7.5g/L agar; Bacto, Sparks, MD) was used to culture *S. mutans* JH1140 ATCC 55676 and *Micrococcus luteus* ATCC 10240 at 37°C.

Table 2.2 Strains and plasmids used in chapter II. All mutations were made in the wild-type strain JH1140. All the plasmids were isolated from *E. coli* DH5α cells.

Strains used	Plasmid Intermediate	Description	References
S. mutans			
JH1140 ATCC 55676		Wild-type bacteriocin producing strain	Strain (124)
lanA:IFDC2	pIFDC2	Gene replacement strain	Plasmid (139)
Δ(-40-33)	pΔ(-40-33)	8 amino acid(AA) N-terminal truncation	This study
Δ(-40-28)	pΔ(-40-28)	13 AA N-terminal truncation	This study
Δ(-40-24)	pΔ(-40-24)	17 AA N-terminal truncation	This study
Δ(-40-37)	pΔ(-40-37)	4 AA N-terminal truncation	This study
Δ(-36-33)	pΔ(-36-33)	Internal 4 AA truncation	This study
Δ(-32-28)	pΔ(-32-28)	Internal 4 AA truncation	This study
Δ(-27-24)	pΔ(-27-24)	Internal 4 AA truncation	This study
Δ(-23-20)	pΔ(-23-20)	Internal 4 AA truncation	This study
Δ(-7-2)*	pΔ(-7-2)	Internal 6 AA truncation	This study
F(-27)A	pF(-27)A	Single alanine substitution at presumed box	This study
D(-26)A	pD(-26)A	Single alanine substitution at presumed box	This study
V(-25)A	pV(-25)A	Single alanine substitution at presumed box	This study
Q(-24)A	pQ(-24)A	Single alanine substitution at presumed box	This study
Q(-24)InsAla	p Q(-24)InsAla)	Alanine insertion at EDLF motif	This study
E(-23)A	pE(-23)A	Single alanine substitution at EDLF motif	This study
E(-23)InsAla	p E(-23)InsAla	Alanine insertion at EDLF motif	This study
D(-22)A	pD(-22)A	Single alanine substitution at EDLF motif	This study
D(-22)InsAla	p D(-22)InsAla	Alanine insertion at EDLF motif	This study
L(-21)A	pL(-21)A	Single alanine substitution at EDLF motif	This study
Δ(-21)	pΔ(-21)	Single AA truncation at EDLF motif	This study
L(-21)InsAla	p L(-21)InsAla	Alanine insertion at EDLF motif	This study
F(-20)A	pF(-20)A	alanine substitution at EDLF	This study

Table 2.2 Continued

Strains used	Plasmid Intermediate	Description	References
Δ(-20)	pΔ(-20)	Single AA truncation at EDLF motif	This study
Δ(-19)	pΔ(-19)	Single AA truncation at EDLF motif	This study
AALF	pAALF	Double alanine substitution at ED	This study
EDED	pEDED	ED substitution for LF	This study
N+6xHis*	pN+6xhis	6xHis addition at N-terminus	This study
N6xHis*	pN6xhis	6xHis substitution at the N-terminus	This study
M. luteus ATCC 10760		Indicator strain in the differed antagonism assay	(40)
E. coli DH5 α	pCR®2.1-TOPO®	Intermediate cloning host	Invitrogen®

II.5.2 *Mutagenesis leader peptide*

The *S. mutans* genome database and *lan* gene cluster, GenBank/EMBL accession number (AF051560), was used to design primers for the mutagenesis and sequencing work. pIFDC2 (139) is an in-frame deletion (IFD) cassette vector, which uses a highly expressed constitutive promoter to drive the expression of a synthetic operon containing both a positive selection marker (*ermAM*) and a negative selection marker (*-pheS*)* (140). Approximately a 500 base pair (bp) amplification of DNA upstream of *lanA* (primers MutA-UpF and MutA-UpR-IDH) and ~500 bp amplification of DNA downstream of *lanA* (primers MutA-DnF-erm and MutA-DnR) were generated. These DNA fragments were attached to the 5' and 3' end of the IFDC2 cassette, respectively. Transformation of this PCR amplified product with *S. mutans* JH1140 ATCC 55676 generated the *S. mutans* strain Δ*lanA*/IFDC2 (Table 2.2). *S. mutans* natural competence

pathway was used for transforming PCR and plasmid products. Natural competence can be activated using a competent stimulating peptide (CSP) (141). An overnight culture of *S. mutans* strain Δ*lanA*/IFDC2 was diluted to 0.1 OD_{600} and grown to 0.25 OD_{600} before the addition of 2 µL of 10 µg/mL CSP to 200 µL of bacterial suspension. After 30 minutes of incubation, 1 µL of the PCR product of the IFDC2 cassette spanned by upstream and downstream DNA from the structural gene (*lanA*) was added to the cells. After 4-5 hours of incubation, 50 µL of solution was plated on THyex plates containing 15 µg/mL of erythromycin. Colonies that grew in the presence of erythromycin were sequenced to confirm that the IFDC2 cassette replaced the *lanA* gene. This strain was used for subsequent transformations of plasmids containing leader peptide mutations. Leader peptide mutations were introduced into the *lanA* gene by 2-step PCR. The primers used to generate each leader peptide mutation are listed in Table 2.3. The mutations were then inserted into pCR®2.1-TOPO® vector according to the provided protocol. The transformants were sequenced by upstream and downstream primers approximately 300 bp from *lanA* using primers MutAsegF and MutAseqR. *S. mutans* Δ*lanA*/IFDC2 was transformed with the same protocol above with 1 µL of cloned pCR®2.1-TOPO® vector. The transformants of the leader peptide mutants were plated on THyex plates with 4mg/mL of chloro-phenylalanine. Colonies growing in the presence of chloro-phenylalanine represent the loss of the IFDC2 cassette and the insertion of the *lanA* with the expected mutation. Colonies from these plates were identically spotted on THyex and THyex with erythromycin to remove false positives from the screen. Mutants were further confirmed by sequencing.

Table 2.3 Forward and reverse primers used in chapter II.

Primer	Sequence
MutA-UpF-long	GCTTCAATTCTTAAATCTAATTTGAATCAGCTTTTATAAA
MutA-DnR-long	TCGGATCACTATGTAGTAACTCAATGGGATCCATCG
MutAseq-F	GAGGCTAATGGTGGTATTATATTATTG
MutAseq-R	ACCAAGGACTTCTAATAATTGTG
MutA-UpF	GCTTCAATTCTTAAATCTAATTTGAATC
MutA-UpR-IDH	GAGTGTTATTGTTGCTCGGACGAGTACTGGATCGTC
MutA-DnF-erm	GGTATACTACTGACAGCTTCTTGTATAAAAGATTTAGATTGTGCC
MutA-DnR	TCGGATCACTATGTAACTCAA
N-6xHis-F	CATCATCATCATCATCATGAAGTCCTTGGTACTGAAAC
N-6xHis-R	ATGATGATGATGATGATGCATAATATCCTCCTTTTTCATGTG
N+6xHisF	GGAGGATATTATGCATCATCATCATCATCATTCAAACACACAATTATTAG
N+6xHisR	CTAATAATTGTGTGTTTGAATGATGATGATGATGATGCATAATATCCTCC
Δ(-40-33)-F	GAAAAAGGAGGATATTATGCTTGGTACTGAAACTTTT
Δ(-40-33)-R	AAAAGTTTCAGTACCAAGCATAATATCCTCCTTTTTC
Δ(-40-28)-F	GAAAAAGGAGGATATTATGTTTGATGTTCAAGAAGATC
Δ(-40-28)-R	GATCTTCTTGAACATCAAACATAATATCCTCCTTTTTC
Δ(-40-24)-F	GAAAAAGGAGGATATTATGGAAGATCTCTTTGCT
Δ(-40-24)-R	AGCAAAGAGATCTTCCATAATATCCTCCTTTTTC
Δ(-40-37)-F	GAAAAAGGAGGATATTATGTTATTAGAAGTCCTTGGT
Δ(-40-37)-R	ACCAAGGACTTCTAATAACATAATATCCTCCTTTTTC
Δ(-36-33)-F	TATTATGTCAAACACACAACTTGGTACTGAAACTTTT
Δ(-36-33)-R	AAAAGTTTCAGTACCAAGTTGTGTGTTTGACATAATA
Δ(-32-28)-F	CACACAATTATTAGAAGTCTTTGATGTTCAAGAAGATC
Δ(-32-28)-R	GATCTTCTTGAACATCAAAGACTTCTAATAATTGTGTG
Δ(-27-24)-F	CTTGGTACTGAAACTGAAGATCTCTTTGCT
Δ(-27-24)-R	AGCAAAGAGATCTTCAGTTTCAGTACCAAG
Δ(-23-20)-F	GAAACTTTTGATGTTCAAGCTTTTGATACAACAGTA
Δ(-23-20)-R	TACTGTTGTATCAAAAGCTTGAACATCAAAAGTTTC
F(-27)A-F	ACTGAAACTGCTGATGTTCAAG
F(-27)A-R	CTTGAACATCAGCAGTTTCAGT
D(-26)A-F	TGAAACTTTTGCTGTTCAAGAAG
D(-26)A-R	CTTCTTGAACAGCAAAAGTTTCA
V(-25)A-F	AACTTTTGATGCTCAAGAAGATC
V(-25)A-R	GATCTTCTTGAGCATCAAAAGTT
Q(-24)A-F	TTTGATGTTGCAGAAGATCTCT
Q(-24)A-R	AGAGATCTTCTGCAACATCAAA
E(-23)A-F	GTCCTTGGTACTGAAGCTTTTGATGTTCAAGAA
E(-23)A-R	TTCTTGAACATCAAAAGCTTCAGTACCAAGGAC

Table 2.3 Continued

Primer	Sequence
D(-22)A-F	TTTGATGTTCAAGAAGCTCTCTTTGCTTTTGAT
D(-22)A-R	ATCAAAAGCAAAGAGAGCTTCTTGAACATCAAA
L(-21)A-F	GATGTTCAAGAAGATGCCTTTGCTTTTGATACA
L(-21)A-R	TGTATCAAAAGCAAAGGCATCTTCTTGAACATC
F(-20)A-F	GTTCAAGAAGATCTCGCTGCTTTTGATACAACA
F(-20)A-R	TGTTGTATCAAAAGCAGCGAGATCTTCTTGAAC
Q(-24)InsAla-F	TTGATGTTCAAGCTGAAGATCTCT
Q(-24)InsAla-R	AGAGATCTTCAGCTTGAACATCAA
E(-23)InsAla-F	GTTCAAGAAGCAGATCTCTTTG
E(-23)InsAla-R	CAAAGAGATCTGCTTCTTGAAC
D(-22)InsAla-F	GATGTTCAAGAAGATGTCCTCTTTGCTTTTGAT
D(-22)InsAla-R	ATCAAAAGCAAAGAGGACATCTTCTTGAACATC
L(-21)InsAla-F	GAAGATCTCGCATTTGCTTTTG
L(-21)InsAla-R	CAAAAGCAAATGCGAGATCTTC
Δ(-21)A-F	TGTTGTATCAAAAGCGAGATCTTCTTGAAC
Δ(-21)A-R	GTTCAAGAAGATCTCGCTTTTGATACAACA
Δ(-20)A-F	TGTTGTATCAAAAGCGAGATCTTCTTGAAC
Δ(-20)A-R	GTTCAAGAAGATCTCGCTTTTGATACAACA
Δ(-19)A-F	TTCAAGAAGATCTCTTTTTTGATACAACAGATAC
Δ(-19)A-R	GTATCTGTTGTATCAAAAAAGAGATCTTCTTGAA
AALF-F	ACTTTTGATGTTCAAGCAGCTCTCTTTGCTTTTGAT
AALF-R	ATCAAAAGCAAAGAGAGCTGCTTGAACATCAAAAGT
EDED-F	GATGTTCAAGAAGATGAGGATGCTTTTGATACAACA
EDED-R	TGTTGTATCAAAAGCATCCTCATCTTCTTGAACATC

II.5.3 *Deferred antagonism assay*

The deferred antagonism assay was performed as previously reported (40). *S. mutans* wild-type and mutant strains were grown overnight in liquid THyex culture. The next morning, the culture was diluted to 0.1 OD_{600} and allowed to grow to a mid-logarithmic phase. The culture was then diluted to 0.05 OD_{600} before spotting 2 µl of the bacterial suspension on fresh pre-warmed THyex plates. A duplicate of triplicate spots

were tested for each strain with wild-type and Δ*lanA* serving as positive and negative controls, respectively. The plates were incubated 18 hours at 37°C in a candle jar. The next day the bacterial colonies were heat killed at 65°C for one and a half hours and then cooled to 37°C. *M. luteus* from a fresh overnight plate was used to inoculate pre-warmed THyex broth and grown at 37°C to a mid-logarithmic phase. The culture was then diluted to 0.2 OD_{600} and diluted 25-fold in pre-warmed (42°C) top agar. 5 mL of top agar containing the bacterial suspension was then poured onto each heat-killed plate and incubated overnight at 37°C. The area for each zone of inhibition was calculated and compared to wild-type zones of inhibition. The activity of the purified variants were determined using the same conditions for overlaying the indicator strain, in which 5 µL of the extracted variants were spotted on the pre-warmed THyex plates after being overlayed with *M. luteus*. Student t-test was the statistical method used determine significance ($p<0.05$).

II.5.4 *Isolation of mutacin 1140 leader peptide variants*

Mutacin 1140 and variants of mutacin 1140 were isolated as previously reported (142). A modified THyex media was used as the fermentation media for inoculation. The media contained 30g/L Todd Hewitt, 3g/L yeast extract, 1 g/L NaH_2PO_4, 0.2 g/L Na_2HPO_4, 0.7 g/L $MgSO_4$, 0.005 g/L $FeSO_4$, 0.005 g/L $MnSO_4$, and 0.3% agar. 500 mL of the semi-solid fermentation media was placed in a 1 L glass beaker and stab inoculated using an inoculating needle. The inoculum was placed at 37°C for 72 hours,

and immediately frozen at -80°C. The media was then thawed in a 55°C water bath for 1 hour. The inoculum was then placed in 250 mL centrifuge bottles and centrifuged at 20,000g for 30 minutes. The collected supernatant was pooled, mixed with chloroform at a 1:1 ratio, and shaken vigorously. The mixture was centrifuged again at 20,000g for 30 minutes. The phase between the aqueous and chloroform layers was collected and allowed to dry overnight. The dried precipitate was resuspended in 35% acetonitrile and tested by deferred antagonism assay for activity. The crude extract was run on either a semi-prep C18 column (Agilent® ZORBAX, ODS, C18, 5µm, 4.6x250mm) or analytical column as previously reported (40). CDAP (1-cyano-4-dimethylaminopyridinium tetrafluoroborate) and Tris [2- carboxyethyl] phosphine (TCEP) was used to determine whether the isolated products had any free thiols resulting from a lack of cyclase activity (143). We followed the procedure as has been previously reported by Kluskens et al. (134) with slight modification. We used 0.1 N hydrochloric acid to dissolve CDAP. As a positive control, we used an extended analog of the chemotactic peptide resact (LRGGGVCGPAGTVCGYGGG-NH2) (144). The purified products were confirmed by mass on a Shimadzu® MALDI-MS on both linear and reflectron modes.

CHAPTER III:

BIOSYNTHESIS AND TRANSPORT OF THE LANTIBIOTIC MUTACIN 1140 PRODUCED BY *STREPTOCOCCUS MUTANS*[*]

III.1. Synopsis

Lantibiotics are ribosomally synthesized peptide antibiotics composed of an N-terminal leader peptide that is cleaved to yield the active antibacterial peptide. There have been significant advancements in molecular tools that can be used in *S. mutans* that promote the study of lantibiotic biosynthesis in this bacterium. Herein, we further our understanding of leader peptide sequence and core peptide structural requirements for the biosynthesis and transport of the lantibiotic mutacin 1140. Our study on mutacin 1140 biosynthesis shows a dedicated secondary cleavage site within the leader peptide, and the dependency of transport on core peptide PTMs. The secondary cleavage site on the leader peptide was found at the (-9) position, which occurs prior to the core peptide being transported out of the cell. The coordinated cleavage at the (-9) position was absent in the *lanT* deletion strain, suggesting that core peptide interaction with LanT transporter enables the uniform cleavage at the (-9) position. Following transport, the LanP protease was found to be tolerant to a wide variety of amino acid substitutions at the primary leader peptide cleavage site with the exception of arginine at the -1 position. Several leader and core peptide mutations produced core peptide variants that had

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intermediate stages of PTM enzyme modifications supporting the concept that PTM enzyme modifications, secondary cleavage, and transport are occurring in a highly coordinated fashion.

III.2. Introduction

Lantibiotics are a class of ribosomally synthesized peptide antibiotics produced by Gram-positive bacteria such as *Lactoccocus lactis* and *S. mutans* (9, 151). Lantibiotics are characterized by the presence of posttranslational modifications, such as dehydrated residues and lanthionine rings. The modified residues Dha and Dhb are formed from dehydration of serines and threonines, respectively. The cyclization between a cysteine and either a Dha or Dhb forms a lanthionine or methyllanthionine ring, respectively. The biosynthetic gene cluster contains all the genes necessary to produce a lantibiotic. The *lan* operon for class I lantibiotics contains genes encoding for the lantibiotic peptide (*lanA*), the dehydratase (*lanB*) responsible for the dehydration of serine and threonine residues, the cyclase (*lanC*) responsible for the stereo-specific formation of the thioether linkages, and an ABC-like transporter (*lanT*) for the export of the modified peptide. The mutacin 1140 operon also contains an additional gene called *lanD*, which is essential for the C-terminal decarboxylation of the core peptide forming an AviCys residue (152). Lantibiotics contain a leader peptide sequence that is cleaved following posttranslational modifications by a dedicated protease called LanP (121, 122). The substrate specificity of LanP varies among lantibiotics (123). In most lantibiotic systems, LanP is believed to be associated with the extracellular leaflet of the

membrane and cell wall (153). Therefore, cleavage does not occur until mutacin 1140 is transported out of the cell by LanT.

The lantibiotic mutacin 1140, produced by *S. mutans* JH1140, has been shown to have a similar mechanism of action as another class I lantibiotic called nisin. Mutacin 1140 and nisin have structurally similar rings A and B (Figure 3.1A) and both nisin and mutacin 1140 bind and abduct lipid II (24, 50-52, 136, 154). Mutacin 1140 has also been shown to be bactericidal to pathogenic bacteria such as MRSA with no significant development of resistance (10). Mutacin 1140 is one of a small group of peptides that is currently under preclinical development for the treatment of Gram-positive infections (155). Many studies have revealed that lantibiotics are structurally diverse and have activity differences to nisin. For instance, mutacin 1140 is a class I lantibiotic like nisin. However, the core peptide of mutacin 1140 is 12 amino acids shorter than nisin. Mutacin 1140 has the same ability to bind to lipid II, but it does not form bacterial membrane pores as nisin (24, 52).

The leader peptide sequences of lantibiotics belonging to the same class generally share structural motifs related to their function. The mutacin 1140 leader peptide sequence is unique among class I lantibiotics in that it has little primary sequence similarities (Figure 3.1B). However, the leader peptide is similar in structure to mutacins produced by other *S. mutans* strains (124, 142, 156). Furthermore, there are considerable sequence differences in leader peptides among lantibiotics with similar core peptide structure. The leader sequence is between 19 to 48 amino acid residues long (123, 127). Due to the vast difference among leader sequences, studies have been

inconsistent with its actual function during lantibiotic biosynthesis. It has been suggested that the leader sequence is a signal for transport, inactivates the peptide to protect the producing strain, and provides recognition for PTM enzymes (117, 128, 157). There are conserved motifs found in some leader peptides, which provide a reasonable basis for the classification of lantibiotics. The common motif found in class I lantibiotics is the F(N/D)LD box (Figure 1B), which some studies show is important for the maturation of the core peptide antibiotic (126-128). Class II lantibiotics contain a GG or GA which is used as a cleavage site for the removal of the leader peptide from the core peptide and contain an upstream EV and/or EL conserved sequence motif (158, 159). Amino acid substitutions in the conserved regions within the leader peptide have shown that the composition of these common motif are important for the maturation of the core peptide, while a vast majority of the leader peptide does not have any amino acid sequence specificity (127, 128, 132, 160). Solution NMR studies have shown that the leader sequence lacks secondary structure (161), while the co-crystal structure of NisB in complex with the substrate peptide NisA shows an antiparallel β-strand (102). It is likely that the leader peptide remains unstructured until it binds to the PTM enzymes. However, additional studies are needed to verify this assumption. Experimental studies suggest that the leader peptide is important for interacting with the dehydratase LanB, cyclase LanC, and transporter LanT (129, 130). However, these studies are challenged by the failure to distinguish between which of the proposed leader peptide functions that are affected by the changes in amino acid residues.

Mutacin 1140 biosynthesis shares some important features with other lantibiotic systems, however, there are considerable differences in the biosynthesis between mutacin 1140 and nisin. In this study, we have identified a secondary cleavage site within the leader peptide. We have also shown relaxed substrate specificity for the LanP protease and that primary cleavage is not dependent on leader peptide sequence. Observations were made from several leader and core peptide mutations that supports the concept that the core peptide is not being shuttled between PTM enzymes and transporter by the leader peptide. Mutacin 1140 biosynthesis is a dynamic process in which PTM enzyme modifications and transport are occurring in a highly coordinated fashion, supporting the hypothesis that leader peptide binding at a single locus is sufficient for coordinating PTM enzyme modifications and transport.

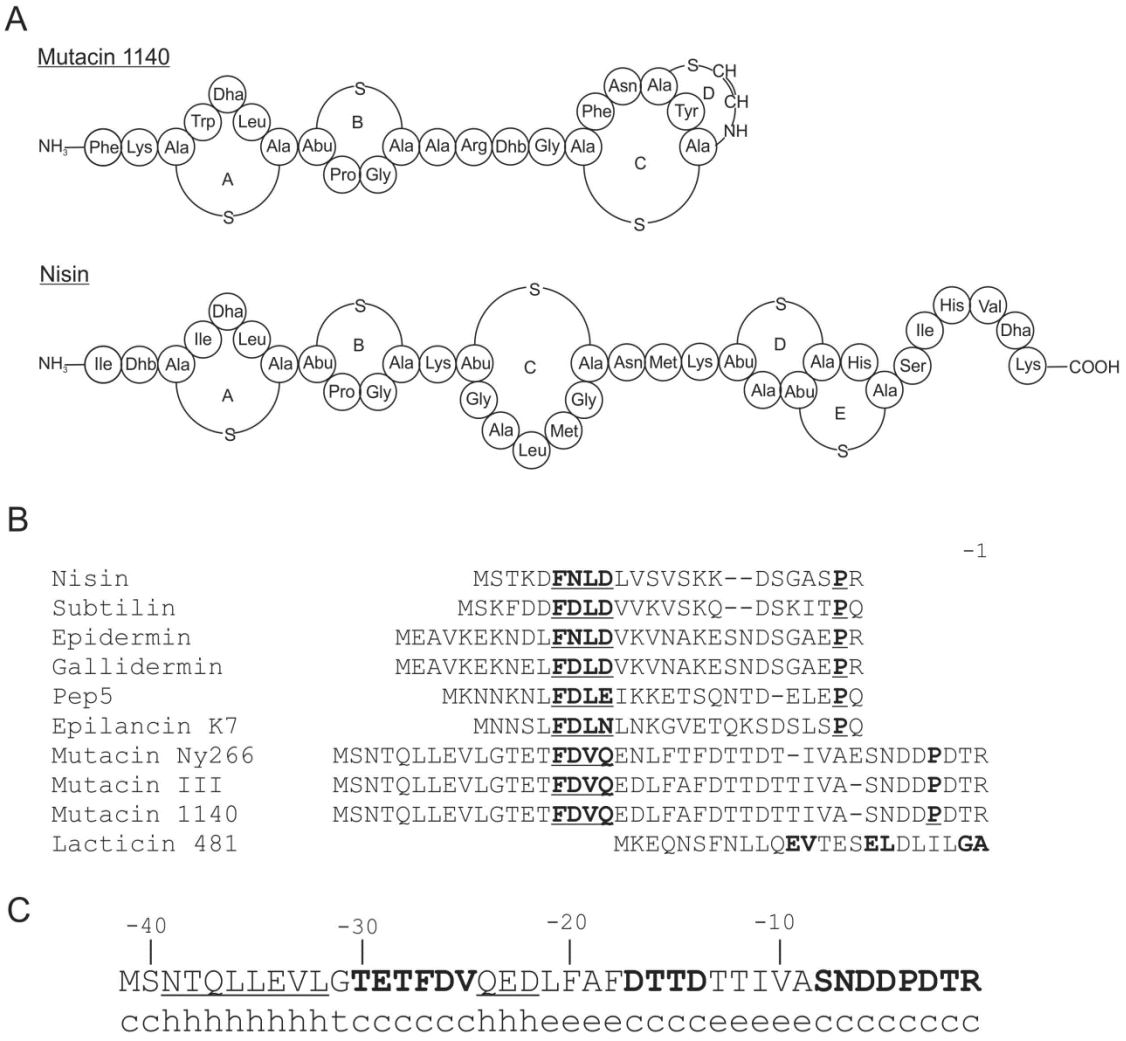

Figure 3.1 Structural elements commonly found in class I lantibiotics **A**) Covalent structures for nisin and mutacin 1140 with the lanthionine rings labeled from N- to C-terminus. **B**) Leader sequence alignments of structurally related Class I lantibiotics; i.e. nisin produced by *Lactococcus lactis*, subtilin produced by *Bacillus subtilis*, epidermin produced by *Staphylococcus epidermidis*, gallidermin produced by *Staphylococcus gallinarium*, Pep5 produced by *Staphylococcus epidermidis*, epilancin K7 produced by *Staphylococcus epidermidis*, mutacin Ny266 produced by *S. mutans* Ny266, mutacin III produced by *S. mutans* UA787, mutacin 1140 produced by *S. mutans* JH1140, and for comparison a class II lantibiotic lacticin 481 produced by *Lactococcus lactis* (23, 142, 145-150, 156). The F(N/D)LD box and C-terminal proline in class I lantibiotics is underlined and in bold print in the sequences. **C**) Secondary structure prediction using SOPMA for mutacin 1140 leader peptide; h (alpha helix), e (extended strand), c (random coil), t (beta turn). Alpha helical regions are underlined, while random coils are in bold in the leader peptide sequence.

III.3. Results

III.3.1 *Specificity of the MutP protease*

We have previously reported that substitutions at the +1 position of the core peptide led to an increase in bioactivity or did not significantly reduce bioactivity (40). This suggests that the presence of F(+1) is not essential for cleavage by LanP. Alignments of the cleavage site of class I lantibiotics show a basic amino acid, such as arginine, at the (-1) position of the cleavage site and a conserved proline at (-2) or (-4) position (Figure 3.1B). Mutations were made to determine whether there is a recognition motif for LanP upstream of the cleavage site. Secondary structure analysis using SOPMA (131) predicts that the N-terminal end of the leader peptide (region (-32) to (-39)) is an alpha helix, while the C-terminal end (region (-1) to (-8)) is a random coil (Figure 3.1C). Interestingly, the (-9) to (-20) region of mutacin 1140 leader peptide is primarily predicted to be an extended strand, which is in agreement with the secondary structure of nisin leader peptide in the co-crystal structure of NisA and NisB (102). Given the lack of predicted secondary structure at the C-terminal end of the leader peptide, LanP protease recognition would presumably be dependent on structural elements upstream or downstream of the primary cleavage site. First, alanine substitutions were made to determine the importance of the amino acids from the (-7 to -1) position of the leader peptide (Figure 2A and 2B). The T(-2)A resulted in a slight reduction in activity, while the other alanine substitutions in residues (-7-3) had no statistical significant decrease in activity. The small change or lack of a statistically reproducible reduction in activity indicates that the region upstream of the cleavage site

does not have a strict motif for proteolytic activity. Interestingly, the substitution of the proline for an alanine at the (-4) position did not result in a decrease in bioactivity. The presence of a proline near the cleavage site is conserved among other class I lantibiotics and the lack of any change in bioactivity with the alanine substitution in this region suggests that the proline is not important for biosynthesis of mutacin 1140. We subsequently tested an alanine substitution at the R(-1) position. This mutation resulted in approximately a 90% loss in bioactivity (Figure 3.2B). The R(-1)A mutation signifies the importance of arginine at this position for LanP activity. The alanine substitutions (-7-2) were also tested in the background of a *lanP* deletion strain to determine whether another protease can cleave the leader peptide. All the substitutions in the *lanP* deletion strain exhibited no activity against the indicator strain *M. luteus*, but activity was restored with the addition of trypsin to the top agar. Removal of the leader peptide using trypsin in a LanP deficient strain is possible given the presence of an Arg at the (-1) position of the leader peptide and the core peptide resistance to trypsin cleavage at the Lys2 and Arg13 position. Interestingly, the bioactivity was significantly larger in the Δ*lanP* strain when trypsin was used to remove the leader peptide. HPLC of the extracted lantibiotic from the Δ*lanP* strain does yield at least a 2-fold increase in purified product (Figure 3.3). The histidine substitution of residues at the (-7-2) position resulted in an ~85% reduction in activity, but did not completely remove all activity. This observation suggests that the LanP is still able to process the removal of the leader peptide containing six histidine substitutions, albeit, at a much lower efficiency. Furthermore, product could not be isolated from the culture broth, suggesting that biosynthesis or

transport of the product is drastically reduced with histidine substitutions of residues at the (-7-2) position. A substitution of histidine at the (-1) position of the leader peptide was evaluated. A histidine side chain has a pK = 6.0 and would predominantly be neutral at the physiological pH of the medium. We introduced this mutation to determine whether the positive charge within the (-1) position was essential for activity and figured this idea could be tested by changing the pH of the growth medium. Testing media with lower pH was not needed since there was no significant change in the bioactivity of the R(-1)H mutation in the medium with the neutral pH. However, we cannot rule out the possible need for a positive charge at the (-1) position, given that the lactic acid production by *S. mutans* could lower the pH around the bacterial colony enabling the histidine to be charged. This data does show that the LanP protease is tolerant to a wide variety of amino acid substitutions upstream of the primary leader peptide cleavage site and that R(-1) is not essential for the removal of the leader peptide.

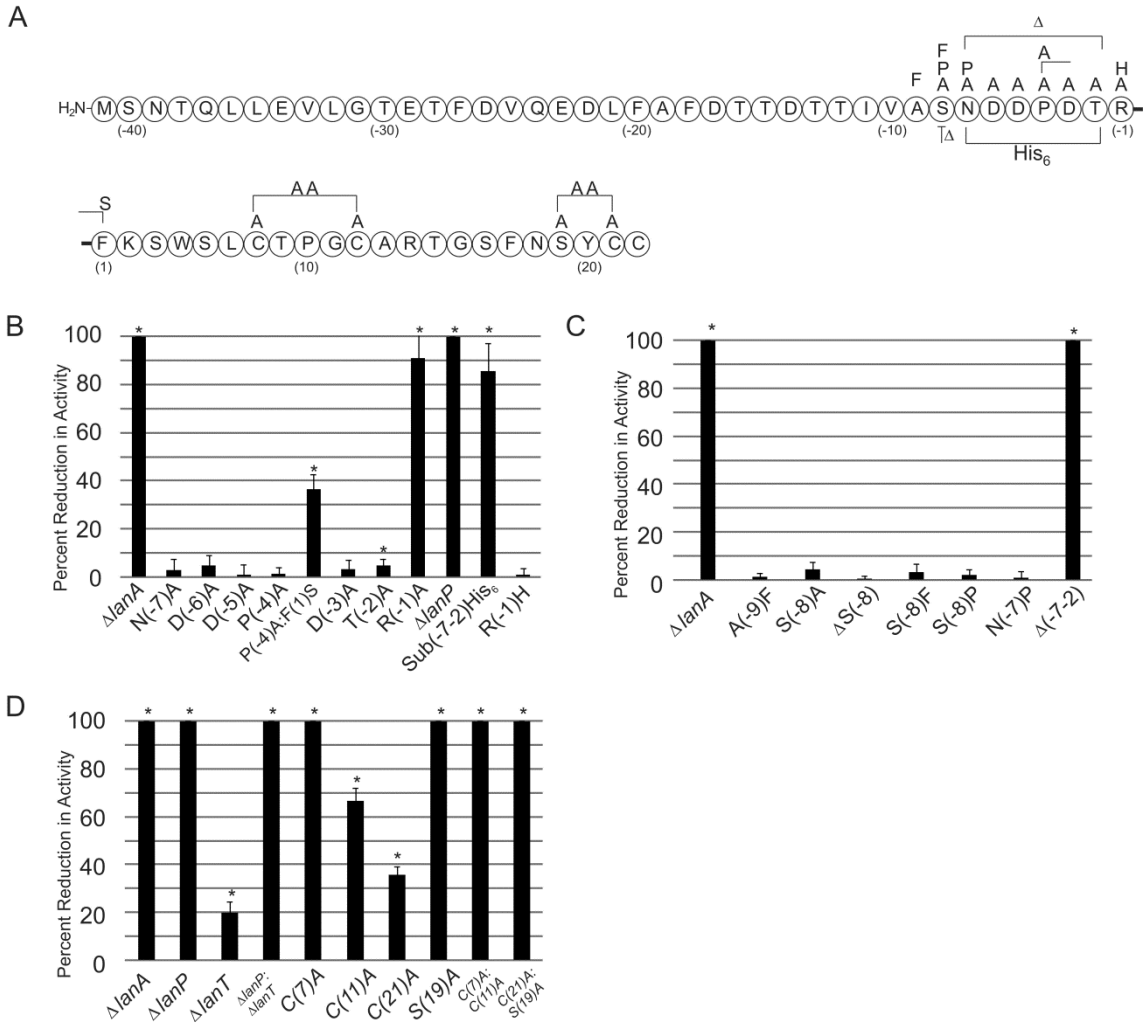

Figure 3.2 Identification of structural elements within the mutacin 1140 leader and core peptide that is important for bioactivity. **A**) Covalent structure representation of the mutations made on the leader peptide. Bioactivity for leader and core peptide mutants were measured as the percent difference in the zone of inhibition between wild-type and the mutant strains. Δ*lanA* strain was used as a negative control for bioactivity in all experiments. The change in activity was measured for: **B**) mutations near the LanP cleavage site, **C**) mutations within the newly discovered cleavage site, and **D**) mutations in the transporter *lanT* and core peptide regions responsible for ring formation. For each mutation, the bioactivity has been compared to the activity of wild-type *S. mutans* JH1140 strain. All the activities are determined in the absence of trypsin in the top agar of the overlays. Statistical method used was Student t-test and the asterisk signifies statistical significance ($p<0.05$).

III.3.2 *Discovery of an additional leader peptide cleavage site*

MALDI-MS of purified products obtained from the culture broth of *S. mutans* Δ*lanP* and R(-1)A strains contained single products with a mass of 3165 Da and 3079 Da, respectively (Table 3.1). The peptide masses indicate the attachment of amino acids (-8 to -1) to the fully modified lantibiotic, instead of the whole 41 amino acid leader peptide as expected in the absence of LanP activity. This observation suggests that an additional cleavage site exists internally within the leader peptide between residues A(-9) and S(-8). The absence of any inhibitory activity of these products also demonstrates that the truncated leader peptide does keep mutacin 1140 in an inactive structural state. It has been shown that biosynthesis of nisin in the absence of LanP produces partially cleaved leader peptide products (162, 163) with full length leader peptide being the main product (164). Unlike nisin, only a single truncated leader peptide product is observed for mutacin 1140 leader peptide. To further understand the importance of the new cleavage site, point mutants were made at the new cleavage site in the *S. mutans* Δ*lanP* strain. This was done in order to prevent cleavage at the primary cleavage site R(-1). The S(-8) or A(-9) were substituted by either an alanine or phenylalanine (Figure 3.2A and 3.2C). The switch to a smaller or bulkier side chain did not affect secondary cleavage activity nor did it affect the bioactivity of the mutant strains in the deferred antagonism assay when trypsin was added (Figure 3.2C). The masses were determined for the isolated mutant products and they still indicated the attachment of the 8 amino acid leader peptide (Table 3.1). Deletion of S(-8) yielded two products with a mass of 3063 Da and 2948 Da, which correspond to partial cleavage at N(-7) and D(-6) positions,

respectively. To further determine whether the cleavage is absolutely necessary, proline mutations were made at either S(-8) and N(-7) positions. Proline mutations will alter the position of neighboring amino acids by introducing a turn in the peptide structure. A proline mutation at S(-8) shifted the cleavage site to I(-11) or T(-13) as indicated by the mass of isolated products (Table 3.1). Interestingly, N(-7)P had major product with a cleavage site at I(-11) but also had multiple minor products with cleavage sites ranging from T(-13) to D(-6). P(-4)A only had a single product that had no shift in the cleavage site between A(-9) and S(-8) (Table 3.1). The structural region of the peptide at the P(-4) position is not important for the alignment of the peptide to be cleaved at the new secondary cleavage site. The structural region for aligning the peptide for cleavage presumably occurs upstream of the P(-4) position. We have previously shown that a serine engineered at the (+1) position does not undergo dehydration (40), suggesting that the distance from either the LanP or new cleavage site is important for a dehydration modification. We engineered a P(-4)A:F(1)S mutant to determine if the proline substitution would enable a dehydration at the (+1) position. The masses of the identified peptide products in the wild-type and *lanP* deletion strains were 2205 and 3078 Da, which corresponds to a product that is missing a dehydration. This suggests that the P(-4)A substitution did not promote a dehydration at the (+1) position and that the length from the (+1) position appears to be important for the dehydratase activity. Interestingly, the double mutant resulted in ~35% reduction in activity, whereas P(-4)A and F(1)S individual mutations resulted in no loss in activity. There was no cyanylation of free

thiols by CDAP in the P(-4)A:F(1)S mutant, suggesting that all lanthionine rings were formed by the cyclase.

Given that a deletion of S(-8) shifted the cleavage site downstream to N(-7) and D(-6) positions, we decided to investigate a larger deletion downstream of S(-8) position. The deletion mutant Δ(-7-2) was engineered in the wild-type and in the *lanP* deletion strains. The deferred antagonism assay for each of these strains had no bioactivity (Figure 3.2A, 3.2C, and 3.4A). Products were isolated from the culture liquor of these mutants and were characterized to have two predominant masses of 1936 and 2508 Da. The 2508 Da product corresponds to the mutacin 1140 peptide that has undergone all of the posttranslational modifications with a short leader peptide of S(-2) and R(-1). Interestingly, the 2508 Da product is present in both the wild-type and *lanP* deletion strains. This suggests that LanP is not capable of processing the truncated (SR) leader peptide in this mutant strain. Trypsin was added to the extracted products to determine whether activity could be restored. The trypsin digested product did have inhibitory activity in our bioassay (Figure 3.4A) supporting the notion that all the posttranslational modifications are present within the 2508 Da mutacin 1140 product. The predominant mass of the mutant product is 1936 Da (Table 3.1 and Figure 3.4B and 3.4C). This mass corresponds to a truncated mutacin 1140 peptide that has been cleaved between S(+3) and Trp(+4) position. Furthermore, the mass of the product corresponds to a loss of dehydration on a serine or threonine residue. We have previously shown that a serine engineered at the (+1) position or the P(-4)A:F(1)S strain does not undergo dehydration (40), suggesting that the distance from either the LanP or new cleavage site

is important for a dehydration modification. Therefore, a loss of a dehydration in the truncated core peptide product presumably occurs at the S(+5) position.

The observation of the 2508 Da peptide corresponds to a fully modified core peptide that has two additional amino acids (SR-) from the leader peptide, while the 1936 Da peptide corresponds to a variant that has been cleaved at S(+3) and is lacking a dehydrated Dha or Dhb residue. Given the observation of two distinct cleavage sites in the deletion mutant Δ(-7-2), there is a possibility that the 2508 Da peptide fragment has been cleaved at both the A(-3) and S(+3) positions, but is being held together by the thioether linkage in ring A. The lack of a single dehydration on the 1936 Da fragment enabled us to test this assumption by 2-mercaptoethanol (BME) derivitization of the two products. The lanthionine rings and the unsaturated amino acids Dha, Dhb, and the AviCys residues are susceptible to the addition of BME. The 2508 and 1936 Da peptides have seven and six modifiable positions, respectively. If there is no proteolytic cleavage between the S(+3) and Trp(+4) positions, derivitization of the 2508 and the 1936 Da peptide should both increase by ~78 Da for each addition of BME. If there is a proteolytic cleavage between the S(+3) and Trp(+4) position of the 2508 Da peptide, derivitization would provide a similar mass profile as the derivatized 1936 Da peptide. The latter scheme was observed following BME derivitization (Figure 3.4D). Given the absence of a dehydration and formation of a lanthionine ring in the 1936 Da peptide product, there would be approximately a 16 Da lower mass for each addition of BME to the 2508 Da peptide product. The mass difference of 16 Da is attributed to the lack of dehydration within the 1936 Da fragment and the loss of 2 Da from the formation of a

disulfide linkage between BME and the free thiol on the cysteine within the 1936 Da fragment. The addition of two to six BMEs were observed by MALDI-MS (Figure 3.4D).

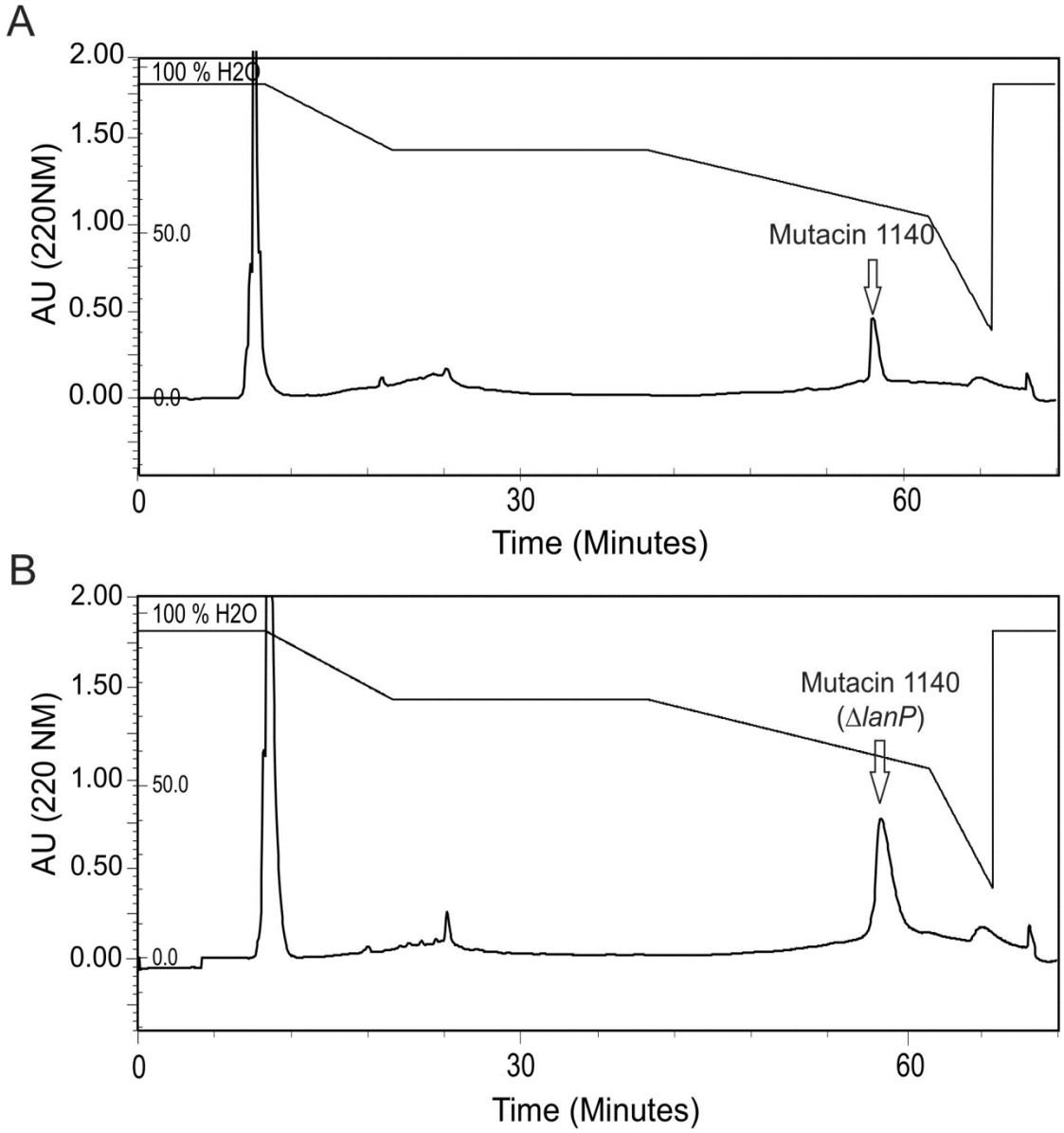

Figure 3.3 HPLC chromatograms of crude extracts obtained from modified THyex media inoculated with **A**) wild-type *S. mutans* JH1140 **B**) and *S. mutans* Δ*lanP*. There is more than a two-fold increase in isolated product from the *S. mutans* Δ*lanP* strain.

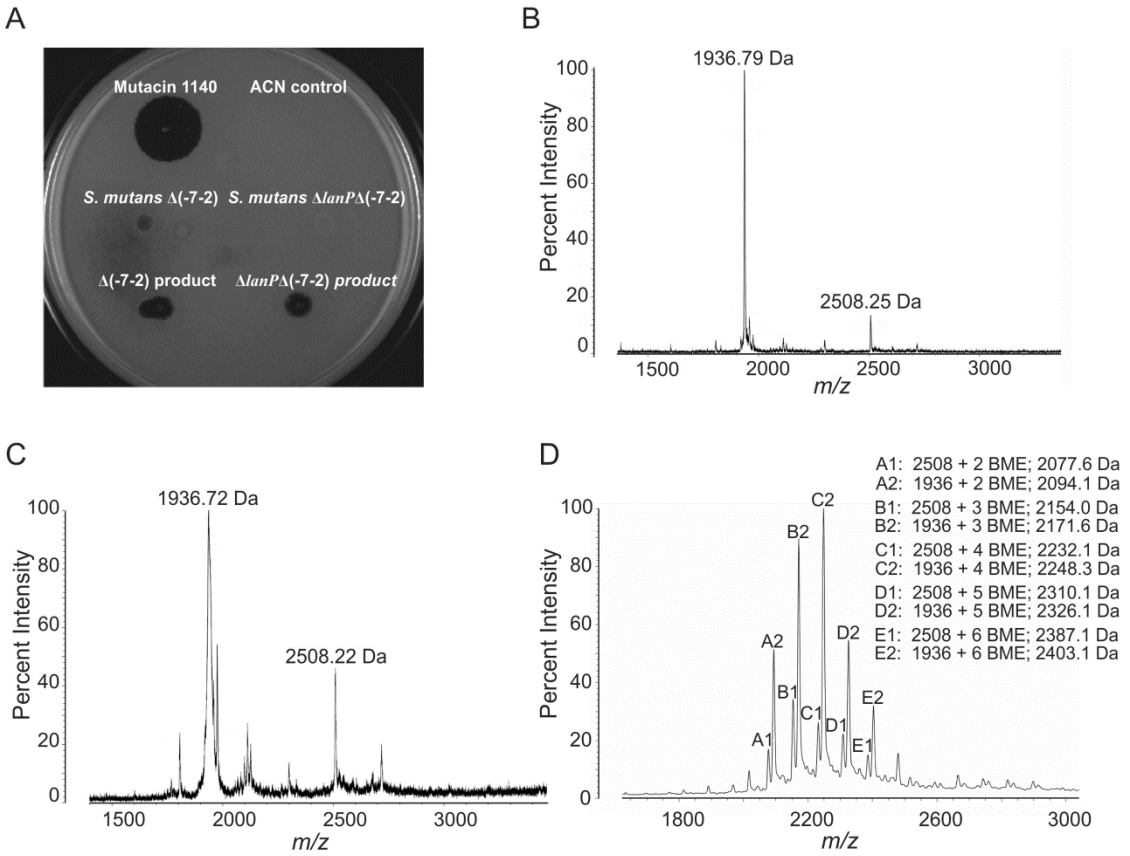

Figure 3.4 Characterization of the leader peptide Δ(-7-2) mutant. **A**) Overlay assay using *M. luteus* as an indicator strain. From top left to bottom right: 5 µL spotted of purified mutacin 1140 (10 µg/mL) in acetonitrile:water (1:1); (negative control) 5 µL spotted of acetonitrile:water (1:1); deferred antagonism assay of *S. mutans* Δ(-7-2); deferred antagonism assay of *S. mutans* Δ*lanP*Δ(-7-2); 5 µL spotted of trypsin digestion of product from *S. mutans* Δ(-7-2); and 5 µL spotted of trypsin digestion of product from *S. mutans* Δ*lanP*Δ(-7-2). **B**) MALDI-MS data of product from *S. mutans* Δ(-7-2). **C**) MALDI-MS data of product from *S. mutans* Δ*lanP*Δ(-7-2). **D**) MALDI-MS data of the 2-mercaptoethanol (BME) derivitization of the isolated products from *S. mutans* Δ(-7-2) strain.

Table 3.1 MALDI-MS data for isolated products from *S. mutans* mutants in chapter III. Some mutant strains produced more than one product. The product masses are listed in the order of highest to lowest relative peak intensities. Some mutant products were not detectable (N.D.) and are listed in the table below.

Strain	Mass (Da)	Leader sequence
S. mutans ATCC 55676	2264.63±1	None
S. mutans Δ*lanP*	3164.50±1	SNDDPDTR
S. mutans A(-9)F Δ*lanP*	3164.50±1	SNDDPDTR
S. mutans S(-8)A Δ*lanP*	3148.50±1	ANDDPDTR
S. mutans S(-8)F Δ*lanP*	3224.60±1	FNDDPDTR
S. mutans Δ(-8) Δ*lanP*	2963.32±1, 3077.42±1	DDPDTR, NDDPDTR
S. mutans S(-8)P Δ*lanP*	3457.90±1, 3660.12±1	IVAPNDDPDTR, TTIVAPNDDPDTR
S. mutans N(-7)P Δ*lanP*	3430.74±1, 3632.75±1, 3317.66±1, 3218.59±1, 3147.51±1, 2963.42±1	IVASPDDPDTR, TTIVASPDDPDTR, VASPDDPDTR, ASPDDPDTR, SPDDPDTR, DDPDTR
S. mutans N(-7)A Δ*lanP*	3121.47±1	SADDPDTR
S. mutans D(-6)A Δ*lanP*	3120.49±1	SNADPDTR
S. mutans D(-5)A Δ*lanP*	3120.49±1	SNDAPDTR
S. mutans P(-4)A	2264.63	
S. mutans P(-4)A Δ*lanP*	3138.46±1	SNDDADTR
S. mutans P(-4)A:F(1)Ser	2204.53±1	N.D.
S. mutans P(-4)A:F(1)Ser Δ*lanP*	3078.36±1	SNDDADTR
S. mutans D(-3)A Δ*lanP*	3120.49±1	SNDDPATR
S. mutans T(-2)A Δ*lanP*	3134.47±1	SNDDPDAR
S. mutans R(-1)A	3079.39±1	SNDDPDTA
S. mutans R(-1)A Δ*lanP*	3079.39±1	SNDDPDTA
S. mutans R(-1)H	2245.58±1	N.D.

Table 3.1 Continued

Strain	Mass (Da)	Leader sequence
S. mutans R(-1)H Δ*lanP*	3145.45±1	SNDDPDTH
S. mutans Δ(-7-2)	1936.23±1, 2507.76±1	None, SR
S. mutans Δ(-7-2) Δ*lanP*	1936.23±1, 2507.76±1	None, SR
S. mutans C6xhis	N.D.	N.D.
S. mutans C(7)A (Ring A)	1699.83±1, 2250.58±1, 2232.57±1	N.D.
S. mutans C(11)A (Ring B)	2250.58±1, 2278.57±1, 2232.57±1	N.D.
S. mutans C(21)A (Ring C)	2232.57±1	N.D.
S. mutans S(19)A (Ring D)	N.D.	N.D.
S. mutans C(7)A:C(11)A	2218.52±1, 1943.17±1, 3100.49±1	SNDDPDTR
S. mutans C(21)A:S(19)A	N.D.	N.D.
S. mutans Δ*lanT*	2264.63±1	N.D.
S. mutans Δ*lanP and* Δ*lanT*	2849.23±1, 3165.50±1, 3448.87±1, 2964.31±1, 2894.24±1, 2637.02±1, 2567.00±1	DPDTR, SNDDPDTR, IVASNDDPDTR, DDPDTR, DPDTR, DTR, TR

III.3.3 *Effects of lanthionine ring mutants on mutacin 1140 transport*

Mutations were engineered to disrupt the formation of ring A, ring B, ring C, ring D, rings AB, and rings CD (Figure 3.2A and 3.2D). Alanine residues were substituted for the cysteines in rings AB and C. Given that the C-terminal cysteine is decarboxylated by LanD, an alanine was substituted for the serine in ring D. Three distinct peptides were isolated from the culture media for the ring A mutant (Table 3.1). A 2250 Da peptide corresponds to the core peptide that has not undergone a dehydration on one of its serine or threonine residues. The 2232 Da peptide corresponds to the core peptide that has undergone dehydration on all of its serine and threonine residues. A truncated core peptide with a mass of 1702 Da corresponds to the loss of FKSW from the N-terminal end. The major peptide mass indentified in the ring B mutant was 2250 Da followed by 2232 Da, corresponding to the lack of a single dehydration within the core peptide and a core peptide that has undergone all dehydrations, respectively (Table 3.1). There was an additional peptide mass of 2278 Da. This corresponds to a core peptide that has undergone all dehydrations and lacks a C-terminal decarboxylation. Ring C mutant had only one peptide product with a mass of 2232 Da, which is the mass of the core peptide that has undergone all dehydrations (Table 3.1). There was no discernible product from the ring D mutant to obtain a mass (Figure 3.5). Formation of ring D appears to be absolutely necessary for transport. Ring A, ring B, and ring C mutants yielded approximately 60%, 90%, and 30% of the product compared to wild-type JH1140 production based on peak volume measurements determined by HPLC chromatograms. The ring AB mutant yielded similar levels as the ring A mutant and the rings CD mutant

had no measurable product as ring D. Interestingly, the ring AB mutant had a minor product with a mass of 3100 Da that included the last 8 amino acids of the leader peptide (Table 3.1). The ring A, ring D, rings AB, and rings CD mutants had no inhibitory activity (Figure 2D). The ring B and ring C mutants had approximately 65% and 35% reduction in activity, respectively.

The bioactivity of the *S. mutans* Δ*lanT* strain was evaluated and it was found to be active in our deferred antagonism assay. The lack of the mutacin 1140 transporter only had approximately 20% reduction in activity. The isolated product from this strain had the predicted 2265 Da mass, suggesting that it had undergone all of the post translational modifications. These results show that there is an alternative transporter within *S. mutans* JH1140 that can transport mutacin 1140. Given the importance of the ring D region within the core peptide for transport (Figure 3.5), the alternative transporter in *S. mutans* JH1140 must also depend on the ring D region for transport. A double deletion strain of the *lanP* and *lanT* was generated to evaluate the nature of the leader peptide. Several peptide products were isolated that correspond to the core peptide containing a leader peptide of two to ten amino acids (Table 3.1). The variation in peptide length is similar to that observed with our proline mutations within the secondary cleavage site.

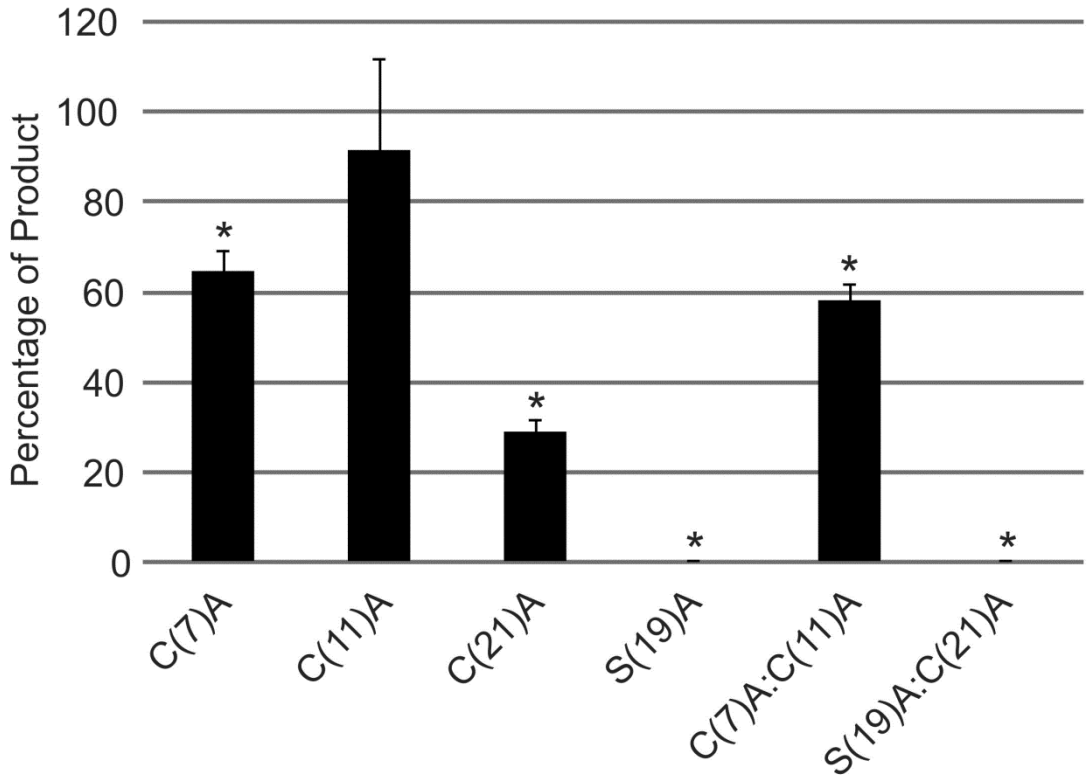

Figure 3.5 Transport efficiencies of mutacin 1140 core peptide variants. Alanine substitutions were made to disrupt ring A (C(7)A), ring B (C(11)A), ring C (C(21)A), ring D (S(19)A), rings AB (C(7)A:C(11)A), and rings CD (C(21)A:S(19)A). Three independent extractions of each mutant strain were characterized by HPLC at 220 nm. Peak volumes of the variants of mutacin 1140 fractions were compared to wild-type strain JH1140 and are represented as percent of product relative to wild-type strain. Statistical method used was Student t-test and the asterisk signifies statistical significance ($p<0.05$).

III.4. Discussion

In this study, we investigated the role of structural regions within the leader peptide and core peptide for biosynthesis. Mutacin 1140 leader peptide sequence is different and longer than other lantibiotics, while the core peptide sequence is similar to nisin, epidermin, and gallidermin. A better understanding of the biosynthesis of all lantibiotics will allow us to advance the use of the PTM enzymes for protein chemistry

applications and the design of novel peptide based therapeutics. We show that secondary cleavage, PTM enzyme modifications, and transport are highly coordinated activities. Terminal core peptide ring formations are essential for transport and the coordinated cleavage at the (-9) position is dependent upon the presence of the LanT transporter. LanP tolerates amino acid substitutions surrounding the primary cleavage site (-1).

A proline usually denotes a site of importance given that they confer breaks in secondary structure and cause turns in peptides or proteins. The P(-4)A mutant resulted in no significant loss in bioactivity. This observation suggests that there is no secondary structure within this region of the leader peptide and that this region is not important for PTM enzyme activity. Furthermore, there was no significant loss in bioactivity or notable interference with the activities of the LanB, LanC, LanD, or LanP PTM enzymes with the removal of the proline, P(-4)A, or the insertion of prolines, S(-8)P or N(-7)P. We only observe a statistically significant slight loss in activity with the T(-2)A substitution. However, bioactivity and mass of the isolated T(-2)A product show that it has undergone all dehydrations and decarboxylation. Secondary structure predictions suggest that the C-terminal end of the leader peptide is a random coil. Our mutational study also suggests that this region is functionally unstructured. We believe the unstructured region enables the interaction of the core peptide to the PTM enzyme active sites, while the upstream box motif of the leader peptide is tethered to a PTM enzyme binding site. This is supported by the co-crystal structure of NisA and NisB (102). The distinct leader peptide binding site and the flexibility of leader peptide residues (-1) to (-8) along with the flexibility of the core region enables the core peptide to reach the

active site for glutamate elimination. Interestingly, following the disruption of Ring B formation in mutacin 1140, we saw a variant that contained a C-terminal carboxyl group. This variant suggests that the core peptide was not efficiently processed by the LanD decarboxylase. The inefficiency of decarboxylation activity supports the concept that PTM enzyme modifications are occurring under the dynamics of core peptide interaction to the PTM enzyme active sites guided by coordinated PTMs on the core peptide.

We saw at least a 2-fold increase in the amount of product isolated from the *lanP* deletion strain. One possible explanation for this increase in activity could be attributed to decrease in product association with the bacterial cell. The short leader core peptide fusion may not readily bind to the cell envelope and the lack of binding would facilitate our extraction from the media. Previous studies with mutacin 1140 suggests that a significant amount of the antibiotic is associated with the cell pellet (69). Another possibility that we are exploring is whether the removal of the leader peptide from the core peptide has an inhibitory role in the production of mutacin 1140 or the leader peptide bound to core peptide is an activator for mutacin 1140 production.

The lack of specificity for both the upstream residues (-7-2) and the F(+1) position near the cleavage site indicates that the protease does not require the recognition of specific amino acid sequence near the cleavage site of the leader peptide. The specificity of cleavage by the LanP protease is unknown. It has been suggested that the specificity can come from structural elements within the core peptide, regions farther upstream of the core peptide, or the protease recognizes the tertiary structure of the truncated leader peptide and core peptide. The latter explanation is unlikely given the

substitution P(-4)A did not result in any loss in activity and that this region is predicted to be unstructured. Furthermore, the recognition of a region upstream of the S(-8) position is not likely given that the leader peptide is only eight amino acids given the presence of the secondary cleavage site. Core peptide recognition by NisP for the cleavage of the leader peptide has been suggested for nisin (117, 153, 165), and given the current results, core peptide structure is important for the specificity of mutacin 1140 leader peptide cleavage by LanP. Additional studies will need to be done to determine whether LanP can recognize unmodified core peptide or whether PTM enzyme modifications are important for the recognition. The inability of the LanP protease to process the 2508 Da product's (SR) leader peptide when there was a cleavage at the S(+3) position of Ring A, suggests that the opening of Ring A prevented LanP cleavage. This is also supported by the observation in the *lanT* and *lanP* deletion strains, there was a (TR) leader peptide variant in the *lanT* and *lanP* deletion strain that was not present in the *lanT* deletion strain, suggesting that the short (TR) sequence was removed by LanP. Therefore, the short leader peptide appears to be recognized by MutP. Furthermore, the major product in the Ring A mutant was a truncated fragment (1699 Da) of the leader peptide. Additionally, a ring AB mutant had a minor product with 8 amino acids of the leader peptide (3100). This data suggests that an intact ring A is important for the specificity of cleavage by LanP or that the ring A mutant product is susceptible to proteolytic cleavage by another protease. It is possible for LanP to function in coordination with the transporter LanT and that this interaction coordinates protease

specificity. However, we have shown that LanP is efficient at processing the removal of truncated leader peptides in the LanT deletion strain.

The (-7-2) deletion resulted in a cleavage within the core peptide S(+3) and a cleavage at the A(-3) position. This cleavage led to the formation of two peptide products with a mass of 1936 and 2508 Da. The presence of the 2508 Da fragment that has also been cleaved after the S(+3) position and held together by a thioether linkage, suggests that dehydration, cyclization, and secondary cleavage are functioning within a highly coordinated fashion. In our proposed model, the formation of the terminal lanthionine rings C and D stabilize an interaction with the transporter (LanT) and that this interaction coordinates the secondary cleavage at the (-9) position by an unidentified protease or proteolytic domain. There are two possible scenarios for the formation of the 1936 and 2508 Da products. The first scenario would involve a highly mobile leader peptide moving back and forth from LanB to LanC enabling the coordination of dehydration and ring formation before coordinating the secondary cleavage that would yield the 1936 and 2508 Da products. A more simplified scenario would involve the binding of the leader peptide at a single position in which the core peptide can be acted upon by the PTM enzymes. This scenario would explain the formation of ring A, followed by the cleavage after S(+3) position and a subsequent lower frequency cleavage occurring after the (-3) position that would free the peptide for transport. The (-7-2) deletion mutation presumably has a slower rate of dehydration at the S(+3) site, which prevents cyclase activity. Without the formation of ring A, the peptide is cleaved and transported yielding the 1936 Da fragment. If ring A forms, the peptide is still

cleaved at the (+3) position within the ring, but is not free to be transported until an additional cleavage occurs after the (-3) position. This data supports the view that secondary cleavage is an integral component to the biosynthesis of mutacin 1140 and that the PTM activity of dehydration, cyclization, secondary cleavage, and transport is a dynamic process acting on the core peptide in a highly coordinated fashion.

Secondary cleavage of the leader peptide was observed in the *lanT* and *lanP* deletion strain, albeit the cleavage appeared to be disordered and led to the formation of a number of peptide products. This suggests that the transporter is involved in coordinating the secondary cleavage site. The isolation of a 1936 Da product also suggests that transport of the core peptide is not directly dependent on the leader peptide. Given that cleavage at the secondary site is independent of LanT, it is logical to assume that the presence of the 1936 Da product is recognized and transported without an attached leader peptide.

The fact that cleavage still occurred regardless of the mutations made in the leader peptide, suggests that the new cleavage site on the leader peptide is an integral component within the biosynthetic pathway of mutacin 1140. The new cleavage site appears to rely more on the length of the leader sequence than the actual amino acid composition with the exception of the proline insertions. It is possible that cleavage aids in the release of the modified peptide from a PTM enzyme before transport or having a truncated leader peptide results in a more energy efficient approach for transporting the modified core peptide. Deletion of *lanT* resulted in the production of fully modified product, but in the background of the *lanT* and *lanP* deletion strain the ordered cleavage

between the A(-9) and S(-8) position was not observed. This is similar to the random cleavage observed in our A(-9)P and S(-8)P mutations. These mutations may interfere with the efficiency of transport of the modified product leaving it susceptible to cleavage by cytoplasmic proteases before being transported. Furthermore, we observed a dependency on ring D formation for the isolation of any product from the mutacin 1140 biosynthesis system. We believe that the interaction of the core peptide with the transporter coordinates the ordered cleavage at the A(-9) position and the absence of LanT leads to a random secondary cleavage within the leader peptide. *S. mutans* JH1140 contains an alternative transporter that efficiently transports these products out of the cell. A lantibiotic transporter in a non lantibiotic producing strain of *S. mutans* GS-5 has been recently reported (166). In this study, the authors demonstrate that the transporter confers protection against two component lantibiotics similar to haloduracin and Smb. It is possible that lantibiotic transporters are more widespread in *S. mutans* given their environmental exposure to lantibiotic and non lantibiotic bacteriocins.

To date, the biosynthesis of only a handful of lantibiotics has been studied. Even though these studies have enriched our understanding of the PTM enzymes, we have barely scratched the surface of understanding how these enzymes coordinate their activities toward the synthesis of a fully functional lantibiotic without the production of a significant amount of side products. Studies within mutacin 1140 biosynthesis system and other lantibiotic biosynthesis systems will promote our understanding of this dynamic process. The structural variants isolated from our mutational study support a simplified model for mutacin 1140 biosynthesis; a model in which the leader peptide

binds to one locus that coordinates the core peptide PTMs. PTMs on the core peptide would occur under a dynamic process, dictated by distance of modifiable residues from PTM enzyme active sites and core peptide structural elements. The products isolated from our mutagenesis study do not support the current consensus within the field for class I lantibiotic biosynthesis, in which the leader peptide shuttles the core peptide to each PTM enzyme and transporter. For instance, the presence of products that were lacking dehydrations or C-terminal decarboxylation following disruption of ring A or ring B formation supports the coordinated activities of PTM enzymes following successive steps of core peptide modifications. Furthermore, a dedicated secondary cleavage site at the A(-9) position is present. Truncation mutations, ΔS(-8) or Δ(-7-2), resulted in a concomitant cleavage toward or within the core peptide, suggesting that the secondary cleavage is coordinated by the binding of the leader peptide upstream of the secondary cleavage site. Furthermore, the truncation of the C-terminal portion of the leader peptide also affected the rate of dehydration at S(+3) position or cyclase activity preventing ring A formation. These results further support the idea that the leader peptide is bound at one location and that the length of the C-terminal portion of the leader peptide is important for promoting an efficient interaction of the core peptide with the PTM enzymes.

The study provides a functional model for the biosynthesis of mutacin 1140 and other related lantibiotics. In this model, the PTM enzymes and transporter are functioning in a highly coordinated fashion, while the binding of the leader peptide to one of the PTM enzymes facilitates the core peptide's interaction with the PTM enzymes

and transporter. Following the secondary cleavage of the leader peptide at the A(-9) position facilitated by modified core peptide interaction with LanT transporter, the remaining portion of the leader peptide is released from the PTM enzyme and the core peptide with the truncated leader peptide is exported. Further studies within mutacin 1140 biosynthetic pathway are underway to determine whether this model will satisfy all of our experimental observations.

III.5. Materials and methods

III.5.1 *Bacterial strains, plasmids and media*

Bacterial strains and plasmids used in this study are listed in Table 3.2. The cloning strain *Escherichia coli* DH5α (Invitrogen, Carlsbad, CA) was cultured at 37 °C on Luria-Bertani (LB) broth or agar. THyex broth (30 g/L Todd Hewitt Broth, 3 g/L yeast extract), THyex agar media (30 g/L Todd Hewitt Broth, 3 g/L yeast extract, 15 g/L agar; Bacto, Sparks, MD) and Top agar media (30 g/L Todd Hewitt Broth, 3 g/L yeast extract, 7.5 g/L agar; Bacto, Sparks, MD) was used to culture *S. mutans* JH1140 ATCC 55676 and *Micrococcus luteus* ATCC 10240 at 37 °C. *S. mutans* ATCC 55676 Δ*lanP* strain was obtained from Oragenics inc.

Table 3.2 Strains and plasmids used in chapter III. Strains with an (*) indicate that mutations were made in both wild-type strain and Δ*lanP* strain. All other mutations were made in the wild-type strain JH1140. All the plasmids came from *E. coli* DH5α cells.

Strains used	Plasmid Intermediate	Description	References
S. mutans			
JH1140 ATCC 55676		Wild-type bacteriocin producing strain	Strain (124)
lanA:IFDC2	pIFDC2	Gene replacement strain	Plasmid (139)
Δ*lanP*	pIFDC2 pΔ*lanP*	*lanP* deletion strain	This study
Δ*lanT*	pIFDC2 pΔ*lanT*	*lanT* deletion strain	This study
Δ*lanP:*Δ*lanT*	pIFDC2 pΔ*lanP* pΔ*lanT*	*lanP and lanT* deletion strain	This study
A(-9)F*	pA(-9)F	Single Phe substitution at internal cleavage site	This study
S(-8)P*	pS(-8)P	Single Pro substitution at internal cleavage site	This study
S(-8)F*	pS(-8)F	Single Phe substitution at internal cleavage site	This study
Δ(-8)*	pΔ(-8)	Single truncation at internal cleavage site	This study
S(-8)A*	pS(-8)A	Single Ala substitution at internal cleavage site	This study
N(-7)P*	pN(-7)P	Single Pro substitution at internal cleavage site	This study
N(-7)A*	pN(-7)A	Single Ala substitution before cleavage site	This study
D(-6)A*	pD(-6)A	Single Ala substitution before cleavage site	This study
D(-5)A*	pD(-5)A	Single Ala substitution before cleavage site	This study
P(-4)A*	pP(-4)A	Single Ala substitution before cleavage site	This study
P(-4)A:F(1)S	p P(-4)A:F(1)S	Ala substitution before cleavage site and Ser substitution after cleavage site	This study
D(-3)A*	pD(-3)A	Single Ala substitution before cleavage site	This study
T(-2)A*	pT(-2)A	Single Ala substitution before cleavage site	This study
R(-1)A*	pR(-1)A	Single Ala substitution at primary cleavage site	This study
R(-1)H*	pR(-1)H	Single His substitution at primary cleavage site	This study
C6xHis*	pC6xhis	6xHis substitution before primary cleavage site	This study
C(7)A	pC(7)A	Disruption of ring A formation	This study
C(11)A	pC(11)A	Disruption of ring B formation	This study
C(21)A	pC(21)A	Disruption of ring C formation	This study
S(19)A	pS(19)A	Disruption of ring D formation	This study

Table 3.2 Continued

Strains used	Plasmid Intermediate	Description	References
C(7)A:C(11)A	pC(7)A:C(11)A	Disruption of ring A&B formation	This study
C(21)A:S(19)A	pC(21)A:S(19)A	Disruption of ring C&D formation	This study
M. luteus ATCC 10760		Indicator strain in the differed antagonism assay	Strain (40)
E. coli DH5 α	pCR®2.1-TOPO®	Intermediate cloning host	Invitrogen®

III.5.2 *Mutagenesis leader peptide*

The *S. mutans* genome database and *lan* gene cluster, GenBank/EMBL accession number (AF051560), was used to design primers for the mutagenesis and sequencing work. pIFDC2 (139) is an in-frame deletion (IFD) cassette vector, which uses a highly expressed constitutive promoter to drive the expression of a synthetic operon containing both a positive selection marker (*ermAM*) and a negative selection marker (*-pheS*)* (140). Approximately a 500 base pair (bp) amplification of DNA upstream of *lanA* (primers MutA-UpF and MutA-UpR-IDH) and ~500 bp amplification of DNA downstream of *lanA* (primers MutA-DnF-erm and MutA-DnR) were generated. These DNA fragments were attached to the 5' and 3' end of the IFDC2 cassette, respectively. Transformation of this PCR amplified product with *S. mutans* JH1140 ATCC 55676 or *S. mutans* Δ*lanP* strain generated the *S. mutans* strain Δ*lanA*/IFDC2 and *S. mutans* strain Δ*lanA*/IFDC2 Δ*lanP* (Table 2). *S. mutans* natural competence pathway was used for transforming PCR and plasmid products. Natural competence can be activated using a competent stimulating peptide (CSP) (141). An overnight culture of either *S. mutans* strain Δ*lanA*/IFDC2 or *S. mutans* strain Δ*lanA*/IFDC2 *lanP* was diluted to 0.1 OD_{600} and grown to 0.25 OD_{600} before the addition of 2 µL of 10 µg/mL CSP to 200 µL of bacterial suspension. After 30 minutes of incubation, 1 µL of the PCR product of the IFDC2

cassette spanned by upstream and downstream DNA from the structural gene (*lanA*) was added to the cells. After 4-5 hours of incubation, 50 µL of solution was plated on THyex plates containing 15 µg/mL of erythromycin. Colonies that grew in the presence of erythromycin were sequenced to confirm that the IFDC2 cassette replaced the *lanA* gene. This strain was used for subsequent transformations of plasmids containing leader peptide mutations. Leader peptide mutations were introduced into the *lanA* gene by 2-step PCR. The mutations were then inserted into pCR®2.1-TOPO® vector according to the provided protocol. The transformants were sequenced by upstream and downstream primers approximately 300 bp from *lanA* using primers MutAsegF and MutAseqR. *S. mutans* Δ*lanA*/IFDC2 was transformed with the same protocol above with 1 µL of cloned pCR®2.1-TOPO® vector. The transformants of the leader peptide mutants were plated on THyex plates with 4 mg/mL of chloro-phenylalanine. Colonies growing in the presence of chloro-phenylalanine represent the loss of the IFDC2 cassette and the insertion of the *lanA* with the expected mutation. Colonies from these plates were identically spotted on THyex and THyex with erythromycin to remove false positives from the screen. Mutants were further confirmed by sequencing.

III.5.3 *Deferred antagonism assay*

The deferred antagonism assay was performed as previously reported (40). *S. mutans* wild-type and mutant strains were grown overnight in liquid culture. The next morning, the culture was diluted to 0.1 OD_{600} and allowed to grow to a mid-logarithmic phase. The culture was then diluted to 0.05 OD_{600} before spotting 2 µl of the bacterial suspension on fresh pre-warmed THyex plates. A duplicate of triplicate spots were tested

for each strain with wild-type and Δ*lanA* serving as positive and negative controls, respectively. The plates were incubated 18 hours at 37 °C in a candle jar. The next day the bacterial colonies were heat killed at 65 °C for one and a half hours and then cooled to 37 °C. *M. luteus* from a fresh overnight plate was used to inoculate pre-warmed THyex broth and grown at 37 °C to a mid-logarithmic phase. The culture was then diluted to 0.2 OD_{600} and diluted 25-fold in pre-warmed (42 °C) top agar. 5 mL of top agar containing the bacterial suspension was then poured onto each heat-killed plate and incubated overnight at 37 °C. Trypsin was used to remove the leader peptide from the core peptide mutacin 1140 by adding it to the top agar at a concentration of 5 µg/ml. The area for each zone of inhibition was calculated and compared to wild-type zones of inhibition. Statistical method used was Student t-test to determine activity differences of each mutant strain with respect to wild-type activity. The activity of the purified variants were determined using the same conditions for overlaying the indicator strain, in which 5 µL of the extracted variants were spotted on the pre-warmed THyex plates after being overlayed with *M. luteus*.

III.5.4 *Isolation of mutacin 1140 leader peptide variants*

Mutacin 1140 and variants of mutacin 1140 were isolated as previously reported (142). A modified THyex media was used as the fermentation media for inoculation. The media contained 30 g/L Todd Hewitt, 3 g/L yeast extract, 1 g/L NaH_2PO_4, 0.2 g/L Na_2HPO_4, 0.7 g/L $MgSO_4$, 0.005 g/L $FeSO_4$, 0.005 g/L $MnSO_4$, and 0.3% agar. 500 mL of the semi-solid fermentation media was placed in a 1 L glass beaker and stab

inoculated using an inoculating needle. The inoculum was placed at 37 °C for 72 hours, and immediately frozen at -80 °C. The media was then thawed in a 55 °C water bath for 1 hour. The inoculum was then placed in 250 mL centrifuge bottles and centrifuged at 20,000 g for 30 minutes. The collected supernatant was pooled, mixed with chloroform at a 1:1 ratio, and shaken vigorously. The mixture was centrifuged again at 20,000 g for 30 minutes. The phase between the aqueous and chloroform layers was collected and allowed to dry overnight. The dried precipitate was resuspended in 35% acetonitrile and tested by deferred antagonism assay for activity. The crude extract was run on either a semi-prep C18 column (Agilent® ZORBAX, ODS, C18, 5µm, 4.6x250mm) or analytical column, as previously reported (40). 2-mercaptoethanol (BME) modifications of mutacin 1140 peptide variants was done as previously reported (24, 29). The lack of PTM cyclase activity would prevent lanthionine ring formation and result in the presence cysteine thiols that can be selectively labeled. CDAP (1-cyano-4-dimethylaminopyridinium tetrafluoroborate) and Tris [2- carboxyethyl] phosphine (TCEP) was used to determine whether the isolated products had undergone all of the PTM cyclase modifications (143). We followed the procedure as has been previously reported by Kluskens et al (134) with slight modification. We used 0.1 N hydrochloric acid to dissolve CDAP. As a positive control, we used an extended analog of the chemotactic peptide resact (LRGGGVCGPAGTVCGYGGG-NH2) (144). The purified products were confirmed by mass on a Shimadzu® MALDI-MS on both linear and reflectron modes.

CHAPTER IV:

EPIDERMIN CLASS LANTIBIOTICS, A NOVEL SCAFFOLD FOR LEAD ANTIBACTERIAL DISCOVERY

IV.1. Synopsis

Mutacin 1140 belongs to the epidermin group of lantibiotics. A C-terminal AviCys residue is derived from the decarboxylation of a terminal cysteine that is involved in one of the lanthionine ring formations. Studies on mutacin 1140 have revealed new insights into the structural importance of the C-terminal AviCys residue. A C-terminal carboxyl analog of mutacin 1140 was engineered. Capping the C-terminal carboxyl group with a primary amine restores bioactivity and affords a novel opportunity to synthesize and test new analogs. For example, a C-terminal fluorescein labeled mutacin 1140 analog trap lipid II into a large lipid II lantibiotic complex, similar to what has been observed *in vivo* for the lantibiotic nisin. A C-terminal carboxyl analog of mutacin 1140 competitively inhibits the activity of native mutacin 1140 and nisin. The presence of a C-terminal carboxyl group prevents the formation of the large lipid II lantibiotic complexes, but does not prevent the binding of the lantibiotic to lipid II.

IV.2. Introduction

Lantibiotics are characterized by their PTMs. Dehydrations of serine and threonine residues into dehydroalanine and dehydrobutyrine residues, respectively, are a common modification found in lantibiotics. These dehydrated residues can be cyclized with cysteines to form thioether bridges, which are called lanthionines (121, 167). Lantibiotics can contain other posttranslational modifications, such as D-alanines in lacticin 3147, β-hydroxy aspartate in cinnamycin, 2-oxopropionyl in lactocin S, and an oxidized lanthionine in actagardine (28, 30, 82, 88). The epidermin group of lantibiotics and other lantibiotic peptides have an AviCys residue at the C-terminal end of the peptide (Figure 4.1A). This residue consists of a decarboxylated cysteine which forms a lanthionine ring. Several of these modifications have been shown to be important for lantibiotic activity, but the importance of the AviCys for activity remains poorly understood in the epidermin group of lantibiotics.

Mutacin 1140, produced by *Streptococcus mutans* JH1140 is a lantibiotic that has shown promise as a potential therapeutic (Figure 4l1B) (124, 168, 169). It has a broad spectrum of activity against Gram-positive bacteria (10). Moreover, mutacin 1140 has been shown to clear *Staphylococcus aureus* infections in rodent models with little associated toxicity (14). The producing bacterium of mutacin 1140 has been engineered into a therapy aimed at preventing dental caries (21). Biosynthetically, mutacin 1140 belongs to the class I epidermin group of lantibiotics and is structurally related to epidermin and gallidermin (149, 170). The first two lanthionine rings, rings A and B, of the epidermin group are referred to as the lipid II binding domain. The lantibiotic nisin

shares structural homology to the lanthionine rings A&B. The latter half of the epidermin and nisin peptide is referred to as the lateral assembly domain, which presumably abducts lipid II into large lipid II/lantibiotic complexes (24, 51).

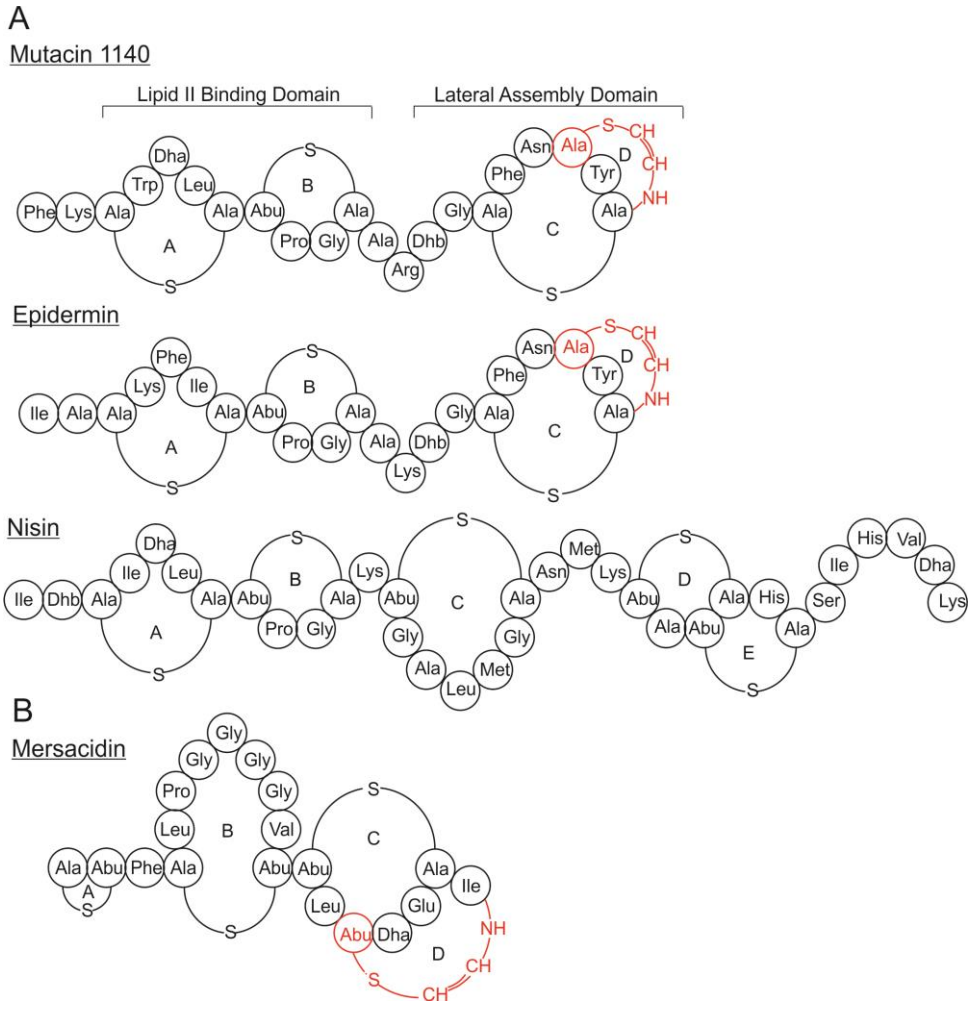

Figure 4.1 Structures of class I and class II lantibiotics containing AviCys residues. **A**) Structures of class I lantibiotics: mutacin 1140, epidermin, and nisin. Dehydrated residues are either Dha or Dhb. The lipid II binding domain of class I lantibiotics consist of the first two lanthionine rings A and B, while the lateral assembly domain consist of the terminal rings. **B**) Structure of the class II lantibiotic, mersacidin, which contains an AviCys. Residues involved in AviCys formation are labeled in red for both classes.

Decarboxylation of a C-terminal cysteine to form an AviCys residue occurs in several metabolites (171). AviCys is present in the class II lantibiotics mersacidin and microbisporicin (171, 172). It is also found in non-lantibiotics, such as cypemycin. Cypemycin contains many of the lantibiotic PTM modifications, but does not form lanthionine rings (173). Lastly, the AviCys residue, also, has been found in the nonribosomal peptide synthetases (NRPS) produced metabolite thiovideramide. The mechanism of AviCys formation for the NRPS peptide maybe different due the nature of its biosynthesis (174). In lantibiotics, decarboxylation is performed by the flavoprotein LanD. This decarboxylase has been shown to be specific for C-terminal cysteines. Furthermore, LanD could not decarboxylate an ethyl-thioether mimic, suggesting decarboxylation occurs before ring D formation (175). Crystal structures for both EpiD, the decarboxylase for epidermin, and MrsD, the decarboxylase for mersacidin, indicates that the enzyme forms a homo-dodecamer (137, 176). Studies on the mechanism of activity suggest that decarboxylation produces an ene-thiol intermediate that promotes terminal ring formation (177). There have been no reports of an isolated carboxylated analog of an AviCys containing lantibiotic, even in an EpiD deletion mutant of epidermin biosynthesis (178). A free carboxyl group analog of the epidermin group of lantibiotics would promote studies aimed at understanding the functional basis for AviCys residues within the lantibiotic.

Chemical modification of lantibiotics offers a novel avenue for the development of new types of therapeutics (179). HOAt/EDC coupling has been achieved for lantibiotics that contain a C-terminal carboxyl group. NVB302, an analog of actagardine

which has undergone phase 1 clinical trials, has a diaminoheptane tail attached to the C-terminus of the lantibiotic (113). Additionally, lantibiotics can be produced through solid-phase peptide synthesis using orthogonally protected lanthionine rings (179, 180). The lack of a C-terminal carboxyl group complicates the further development of the epidermin group of lantibiotics using these methods. We engineered a C-terminal carboxyl analog of mutacin 1140 that promoted studies toward understanding the functional importance of the AviCys residue in the epidermin group of lantibiotics.

IV.3. Results

IV.3.1 *Engineering the production of a c-terminal carboxyl analog of an epidermin group lantibiotic*

A C-terminal carboxyl analog for the epidermin group of lantibiotics has not \been characterized. Several structural analogs of mutacin 1140 were identified when the formation of ring B was disrupted by a C(11)A mutation. The mutation interfered with the formation of the PTMs normally found within mutacin 1140 and one of the peptide analogs had not undergone a C-terminal decarboxylation (32). This observation supported the basis for engineering *S. mutans* JH1140 to produce a mutacin 1140 C-terminal carboxyl analog (mu1140-COOH). Deletion or insertional mutagenesis of *mutD* was done using the IFDC2 gene replacement system (Figure 4.2A) (139). A deferred antagonism assay, using the indicator strain *Micrococcus luteus* ATCC 10240, was performed on the *S. mutans* insertional IFDC2:*mutD* and *S. mutans* Δ*mutD* mutant strains. Neither mutant strains produced clear zones of inhibition (Figure 4.2B),

suggesting that the mutants did not produce a product or that the product was inactive. The culture broth of each mutant was extracted using the same extraction method for wild-type mutacin 1140. These extracts were run on an HPLC, as previously described (40), there was no observable product for the Δ*mutD* strain. The ribosomal binding site (RBS) for *mutP* protease is within the 3' end of the *mutD* gene. Careful consideration was made to leave the RBS in the deletion strain. However, there may be other elements important for the synthesis of downstream products that are not readily apparent. Nevertheless, a single HPLC peak for IFDC2:*mutD* strain was isolated and further characterized (Figure 4.2C). The IFDC2 cassette is under the control of a constitutive lactose dehydrogenase (*ldh*) promoter and it is likely that this promoter facilitates the expression of the downstream genes. The product purified from this mutant was analyzed by MALDI-MS and had a mass of 2310 Da. This mass corresponded to the expected mass of a C-terminal carboxyl analog of mutacin 1140. A MIC assay performed using mu1140-COOH analog against *M. luteus* revealed a 256-fold reduction in activity compared to wild type mutacin 1140. Furthermore, the activity of mu1140-COOH against *Streptococcus pneumoniae* ATCC 27336 was greater than 64 ug / mL, which is more than a 128-fold reduction in activity (Table 4.1). The loss in activity in the MIC assays further corroborates the lack of activity seen in the deferred antagonism assays. The reason for the absence of activity may be attributed to the presence of the carboxyl group or it could indicate that the presence of the carboxyl group has disrupted the occurrence of other PTMs found in mutacin 1140.

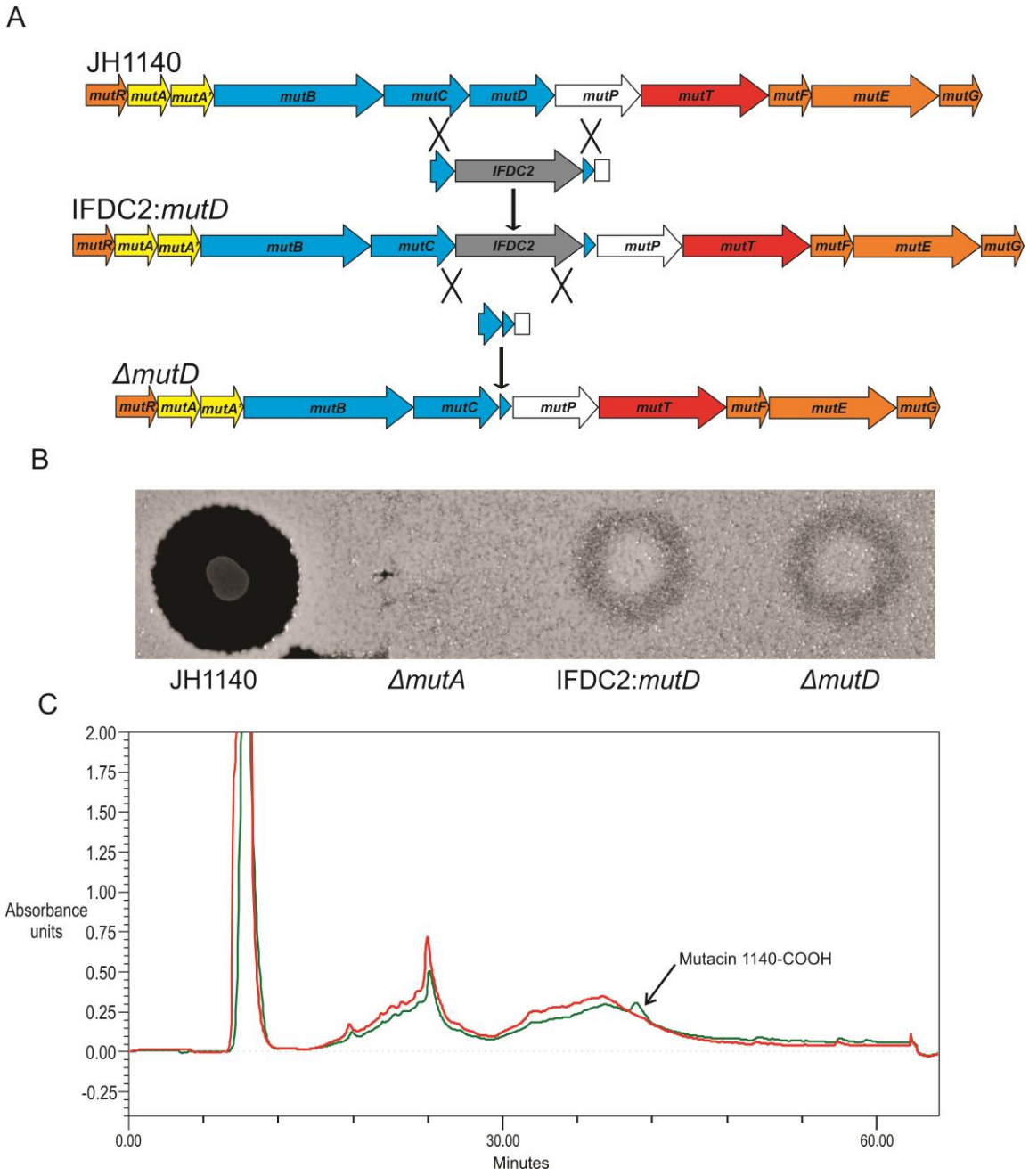

Figure 4.2 Deletion of *mutD* in *S. mutans* JH1140 **A**) Scheme for the deletion of *mutD* by IFDC2 gene replacement. **B**) Deferred antagonism assay against *M. luteus* ATCC 10240 shows no zone of inhibition for either *S mutans* IFDC2:*mutD* or *S. mutans* Δ*mutD*. *S. mutans* JH1140 and *S. mutans* Δ*mutA* were used as a positive and negative controls, respectively. **C**) Purification of *S mutans* IFDC2:*mutD* extracts (green) show a single peak, while there was no observable peak for *S. mutans* Δ*mutA* and Δ*mutD*.

Table 4.1 Mass and activity of chemically modified analogs of mutacin 1140 against select bacteria.

Mutacin 114O analog	Expected Mass (Da)	Observed Mass (Da)	MIC (μg/mL) *M. luteus*	MIC (μg/mL) *S. pneumoniae*
Mutacin 1140	2264.63	2264.63	0.125	0.5
Mu1140 - COOH	2310.65	2310.63	32	>64
Mutacin 1140 – methylamine	2325.72	2325.42	0.125	0.5
Mutacin 1140 – diaminoheptane	2422.88	2421.97	0.25	2
Mutacin 1140 – 4-chlorobenzylamine	2436.25	2435.80	1	8
Mutacin 1140 - 3,4-dichlorobenzylamine	2470.69	2468.26	4	8

MALDI-MS analysis can determine dehydrations due to an observable change in mass. It cannot determine the formation of a lanthionine ring after dehydration, since the PTM does not result in a change in mass of the peptide. Therefore, the formation of the lanthionine rings needed to be assessed by another method. A rapid and straight forward Edman sequencing method was developed by our group in order to distinguish between dehydrated residues and dehydrated residues involved in lanthionine ring formation (29). Dehydrated residues are first hydrated by sodium borohydride before lanthionine ring derivatization by an organothiol compound. This method determines where the lanthionine rings are formed and the location of all the dehydrated residues, thus, elucidating the covalent structure of the lantibiotic peptide. Sodium borohydride

reduction of Dha and Dhb residues results in the formation of alanine and 2-aminobutyric acid, respectively. Subsequent ethanethiol derivatization opens the lanthionine rings, which form either thioethyl cysteines (S-EC) or β-methylthioethyl cysteines (β-M-S-EC). The presence of an S-EC or BM-S-EC residue at the amino acid positions 3, 8, 16, and 19 indicate that the free thiols of upstream cysteines had reacted with their downstream dehydrated residues to form a lanthionine ring. As predicted, alanine and 2-aminobutyric acid were observed in positions 5 and 14, respectively (Figure 4.3). These correspond to the reduction of the Dha5 and Dhb14 residues. S-EC or BM-S-EC residues were observed in the expected amino acid positions 3, 8, 16, and 19 (Figure 4.3), confirming that they were involved in lanthionine ring formations. The observed data suggested that the loss of activity was not due to disruptions to the other PTM's, and that the observed loss in bioactivity of the mu1140-COOH analog was due to the presence of a C-terminal carboxyl group.

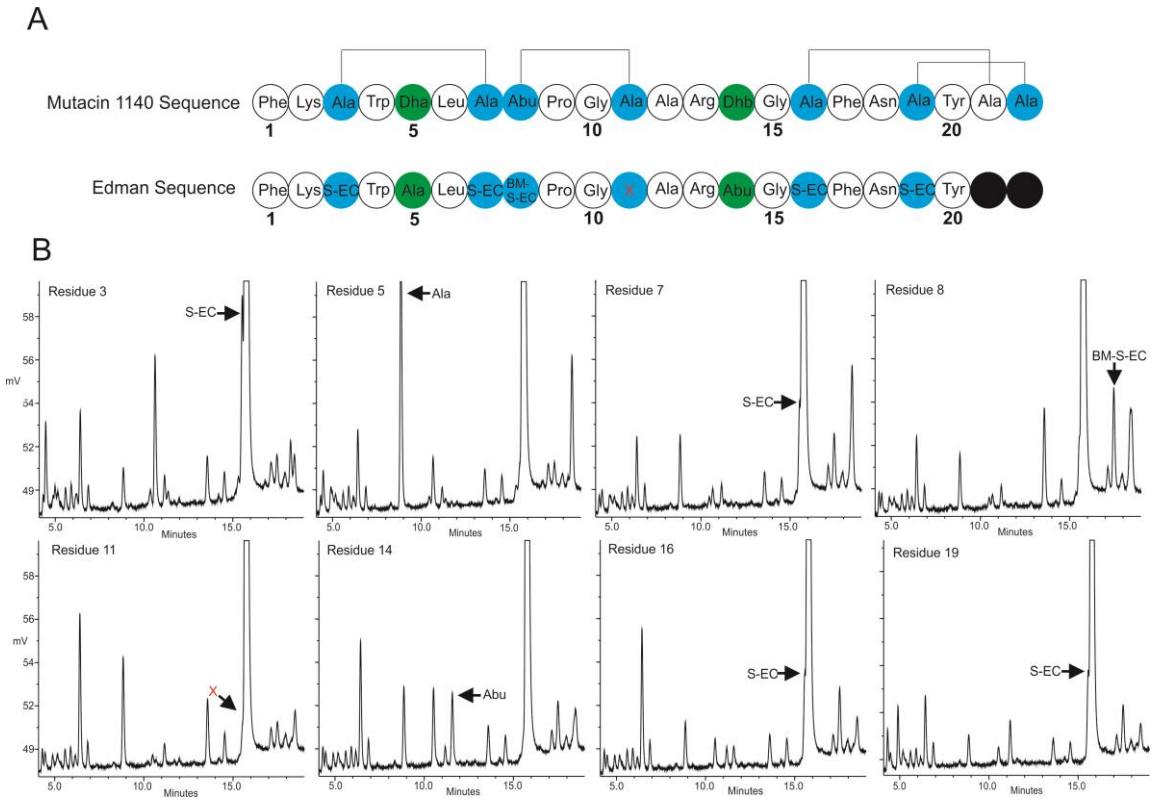

Figure 4.3 Edman sequencing of mutacin 1140-COOH. **A**) After double labeling with sodium borohydride and ethanethiol, a thio-ethyl cysteine (S-EC) or beta-methyl thio-ethyl cysteine (BM-S-EC) is expected at sites of lanthionine ring formation in the Edman sequence compared to mutacin 1140. Blue circles indicate residues expected to form lanthionine rings, and green circles indicate sites of dehydration. **B**) Select Edman sequence spectras for the modified residues indicate full modification of mu1140-COOH. The red X or black circles indicate residues with no signal acquired.

IV.3.2 *Restoration of bioactivity of the c-terminal carboxyl analog of an epidermin group lantibiotic*

Removal of the carboxyl group by MutD was first attempted to confirm that the presence of the C-terminal carboxyl group is responsible for the loss in bioactivity. A C-terminal histidine tag of MutD was constructed and overexpressed in *Escherichia coli*. The purified product on an SDS-PAGE gel had the expected monomer size of 25 kDa,

but subsequent purification through FPLC showed a product that was approximately 200 kDa. A 200 kDa mass is consistent with the formation of a homododecamer, as was previously reported for the epidermin decarboxylase EpiD (176). To determine if the enzyme was active, a reference peptide SFNSYTC, was incubated with MutD for one hour. A mass of 797.67 Da determined by MALDI-MS was observed for the reference peptide compared to 843.69 Da for the unreacted peptide, indicating that the decarboxylase was active (Table 4.2). The mu1140-COOH analog was incubated with MutD for one or ten hours and showed no indication of a mass change by MALDI-MS (Table 4.2). These results suggested that MutD was not capable of decarboxylating the mu1140-COOH analog.

Table 4.2 Mass of mu1140-COOH and control peptide after *in vitro* decarboxylation with MutD.

Substrate	Mass before reaction (Da)	Expected mass (Da)	Observed mass (Da)
SFNSYTC (1hr)	843.69	797.67	797.67
Mu1140-COOH (1hr)	2310.37	2265.37	2310.63
Mu1140-COOH (10hr)	2310.37	2265.37	2310.63

Given that MutD could not remove the C-terminal carboxyl group, the C-terminal carboxyl group was chemically modified and tested to determine whether C-terminal substitutions could restore the bioactivity. EDC coupling of primary amines was used to cap the C-terminal carboxyl group (Figure 4.4). None of the primary amines,

tested on their own, had any activity against the indicator strains used in this study. EDC coupling with methylamine, the smallest primary amine, was first attempted. The conjugation with methylamine yielded a product with a mass of 2321 Da, indicating that the reaction occurred (Table 4.1). Based on the HPLC spectra, the reaction did not yield any other side products and the methylamine product was greater than 95% of the material (data not shown). There was a small amount of unreacted mu1140-COOH analog eluting before the conjugated methylamine product. The methylamine conjugated mutacin 1140 analog had an MIC of 0.125 µg/mL against *M. luteus*, and 0.5 µg/mL MIC against *S. pneumoniae.* These values were the same as the MIC values for native mutacin 1140. These results show that capping mu1140-COOH with a small primary amine restores activity to wild type levels. This data also supports the notion that the presence of a C-terminal carboxyl group is responsible for the reduction in mu1140 activity. We subsequently conjugated diaminoheptane with mu1140-COOH. The activity of the diaminoheptane conjugate was 0.5 µg/mL against *M. luteus* and 2.0 µg/mL against *S. pneumoniae*. The addition of two different chlorinated aromatic rings with mu1140-COOH was also tested. The activity of 4-chlorobenzylamine conjugate was 1.0 µg/mL against *M. luteus* and 2.0 µg/mL against *S. pneumoniae*. For the 3,4-dichlorobenzylamine conjugate, the activity was 1.0 µg/mL and 8.0 µg/mL against *M. luteus* and *S. pneumoniae*, respectively. All the conjugates restored the activity of the mu1140-COOH analog, supporting the synthesis of a library of analogs that can be screened for novel applications.

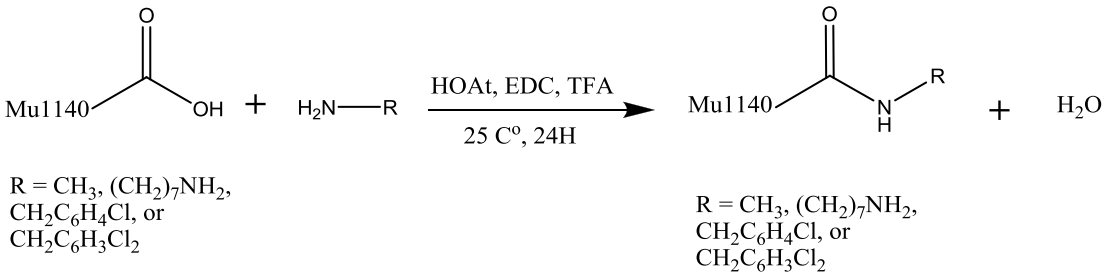

Figure 4.4 Scheme for chemical modification of mu1140-COOH. Mu1140-COOH analog was coupled to various primary amines using HOAt/EDC coupling. Primary amines were chosen based on size and differences in physiochemical properties. Reaction conditions were constant for each substrate, and yields were greater than 80% for each substrate.

Fluorescently-labeled nisin, containing a C-terminal conjugate of fluorescein, forms lipid II patches on the surface *Bacillus subtilis* cells (51). The binding of lipid II by an epidermin group of lantibiotics has been shown in various *in vitro* assays(52), but the bioactivity has never been visualized *in vivo*. This is due to the lack of amenable attachment site for a fluorophore. The free carboxyl group on the mu1140-COOH analog is one such site. Therefore, a C-terminal fluorescein conjugate of mutacin 1140 was evaluated. The product had the expected mass of 2623 Da and was shown to have inhibitory activity on a deferred antagonism assay. For comparison, a C-terminal fluorescein conjugate of nisin was also made as previously described (51). As shown by Hasper et al, *B. subtilis* cells incubated with fluorescein labeled nisin showed large green patches on the cell surface (Figure 4.5). The green patches have been attributed to lipid II abduction and sequestration by nisin from its normal physiological location. Similar to what was observed for nisin, the C-terminal fluorescein-labeled mutacin 1140 produced patterns of fluorescent patches (Figure 4.5). This data supports *in vitro* data from the epidermin group of lantibiotics for lipid II binding and sequestration and also

demonstrates that this group of lantibiotics does sequester lipid II from its normal physiological location for cell wall synthesis.

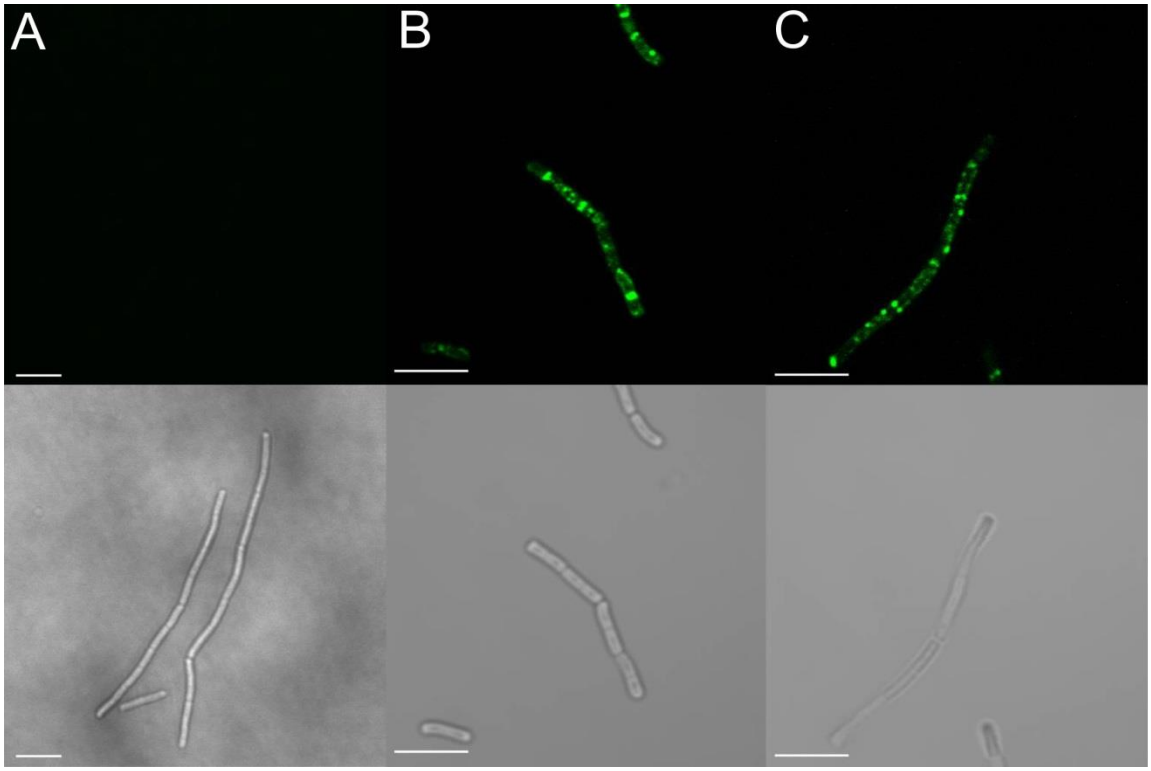

Figure 4.5 *In vivo* localization of mutacin 1140. *B. subtilis* PY79 cells were incubated with various fluorescein conjugated lantibiotics. **A**) A no antibiotic sample was used as a negative control. **B**) Fluorescein conjugated nisin binds and abducts lipid II to form patches as expected. **C**) Fluorescein conjugated mutacin 1140 has a similar localization pattern as nisin. Images were taken using a confocal Olympus microscope using a 100x objective, or a 40x objective for the control.

IV.3.3 *Further elaboration for the loss of activity for the c-terminal carboxyl analog*

With the loss of activity associated with a free carboxyl group in mu1140-COOH, we sought to determine the basis for the loss in activity. The mechanism of action for class I lantibiotics has already been determined to be due to lipid II binding (33, 181, 182). In particular, rings A and B are believed to form a cage-like structure around the pyrophosphate moiety of lipid II (154). The latter half of the peptide is predicted to enhance binding and recruit other lipid II-lantibiotic molecules into a large lipid II-lantibiotic complex (51). Given that lipid II binding is done by the N-terminal rings A and B, it is likely that the loss of activity is due to a loss in the lateral assembly function of the latter half of the peptide. To test for this assumption, we performed a series of experiments to determine whether the mu1140-COOH would bind to lipid II or competitively block lipid II binding of nisin *in vivo*. A TLC plate assay was used to determine if mu1140-COOH could bind to lipid II, as has been previously reported for gallidermin (183). If lipid II migration on the TLC plate is impeded by binding to mutacin 1140 or mu1140-COOH, an iodine stained spot is observed at the origin. No stain was visible for mutacin 1140, mu1140-COOH, and lipid II, when the compounds were spotted alone. However, a stained spot appeared at the origin when mutacin 1140 or mu1140-COOH were spotted with lipid II (Figure 4.6). The mu1140-COOH analog mixed with lipid II showed a faint stain compared to lipid II mixed with wild-type mutacin 1140. The faint staining may be attributed to a weaker association of mu1140-COOH with lipid II than mutacin 1140. If mu1140-COOH is capable of binding to lipid II, but does not inhibit cell growth, it may provide resistance to a bacterium against

mutacin 1140 or nisin. A competition MIC for mutacin 1140 and nisin was then performed against *B. subtilis* and *M. luteus* that were pretreated with mu1140-COOH. *B. subtilis* and *M. luteus* were pretreated with mu1140-COOH at 0.25µg/mL and 0.125µg/mL, respectively, before adding mutacin 1140. Mu1140-COOH competitively inhibited the activity of mutacin 1140 against *B. subtilis* and *M. luteus* by 4-fold (MIC 1.0 µg/mL and 0.5µg/mL, respectively) (Table 4.3). When pretreated with 0.5 µg/mL of mu1140-COOH, the activity of nisin was competitively inhibited against *B. subtilis* and *M. luteus* by 4-fold and 2-fold, respectively (MIC 2.0 µg/mL and 1.0 µg/mL). To further clarify a mechanism of action for the loss of activity in the MIC competition assay, confocal microscopy was used to observe nisin binding as previously described (34). If mu1140-COOH is still binding to lipid II *in vivo*, it should competitively inhibit the binding of fluorescein labeled nisin (Figure 4.7). When the bacteria were pretreated with wild-type mutacin 1140 or mu1140-COOH, no typical *in vivo* fluorescence pattern was observed for the fluorescein labeled nisin (Figure 4.7). However, there was some association of fluorescein labeled nisin with the bacteria, but nothing remotely similar to fluorescein labeled nisin on its own. This may be due to the weaker association of mu1140-COOH to lipid II, which further corroborates the weak association with lipid II observed in the TLC assay. These results suggest that the loss in activity is not due to its inability to bind to the lipid II target, but due to its inability to form the large lantibiotic lipid II complexes.

Figure 4.6 *In vitro* lipid II binding assay. TLC plate assay of mutacin 1140 or mu1140-COOH mixed with lipid II. Binding of lipid II will keep lipid II at the origin. Lipid II or mu1140-COOH by itself was used as a negative control showing no staining. Lipid II and mutacin 1140 was used as a positive control for trapping lipid II at the origin.

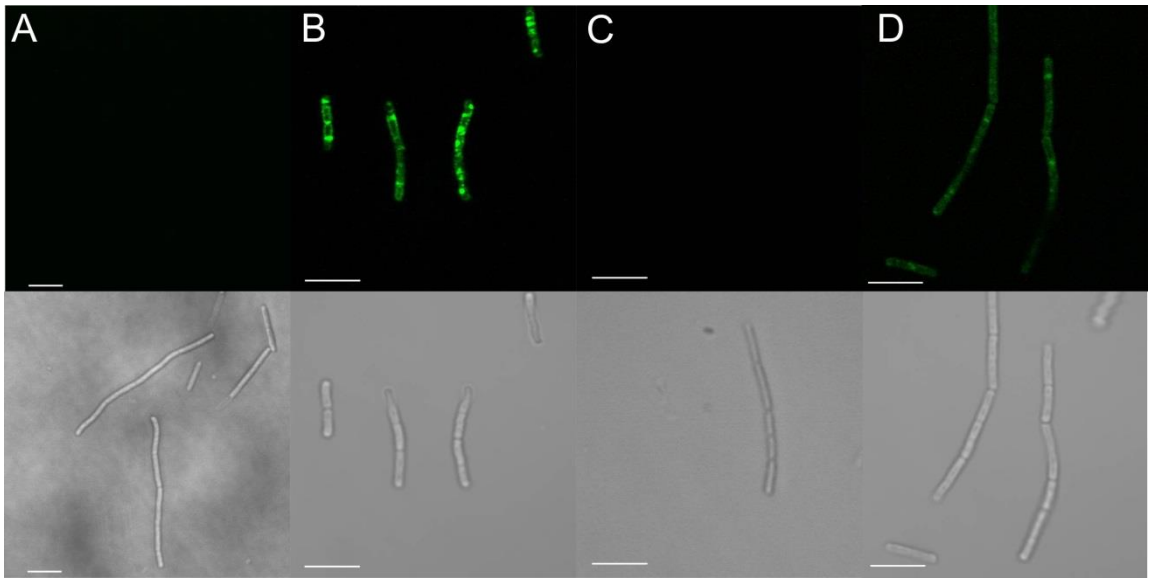

Figure 4.7 Lipid II competition assay of nisin and mu1140-COOH analog. *B. subtilis* cells were treated with mu1140-COOH to compete with nisin in binding to lipid II **A**) Solvent blank (no antibiotic) control. **B**) Fluorescein labeled nisin binds tightly to lipid II to form patches. **C**) Prior treatment of cells with mu1140 prevents binding of fluorescein labeled nisin. **D**) Prior treatment of cells with mu1140-COOH drastically reduces binding of fluorescein labeled nisin. Images were taken using a confocal Olympus microscope using a 100x objective, or a 40x objective for the control.

Table 4.3 Competition MICs of mutacin 1140 or nisin against *B. subtilis* and *M. luteus* preincubated with mu1140-COOH.

Antibiotic	*B. subtilis* MIC (ug/mL)	*B. subtilis* comp. MIC (ug/mL)	*M. Luteus* MIC(ug/mL)	*M. Luteus* comp. MIC (ug/mL)
Mutacin 1140	0.25	1.0	0.125	0.5
Nisin	0.5	2.0	0.5	1.0

IV.4. Discussion

The findings from this study show that decarboxylation in mutacin 1140 is not needed for the other PTM modifications. A fully modified analog of mutacin 1140 without a C-terminal decarboxylation (mu1140-COOH) can be isolated and purified. The bactericidal activity of this analog can be restored by labeling the carboxyl group with a primary amine; a fluorescein conjugated mutacin 1140 can now be synthesized, enabling *in vivo* visualization of mutacin 1140 bound to lipid II target. Lastly, the loss of activity of the mu1140-COOH analog is likely due to the carboxyl group disrupting mutacin 1140 lipid II complex formation and not due to the complete loss of lipid II binding. These data support the need for a lateral assembly mechanism that traps lipid II into a complex for bactericidal activity, a mechanism that is distinct from other lipid II binding antibiotics, *i.e*. vancomycin (136). Furthermore, these data support future studies aimed at generating a C-terminal labeled library of mutacin 1140 analogs that may expand the antibiotic's application or therapeutic use. Our findings have opened a new avenue for the development of novel analogs for the epidermin group of lantibiotics.

A variant of the epidermin group of lantibiotics with a C-terminal carboxyl group has never been isolated. A deletion of *epiD* showed no activity, but no discussion of an isolated product was mentioned (178). Presumambly, this is due to the lack of a product as was observed in the *mutD* deletion strain. Increasing activity or developing new uses for existing lantibiotics has been a goal of many researchers in the field. This has been achieved through a variety of methods, such as amino acid substitutions and the use of non-proteogenic amino acids (53, 98, 179). Semi-synthetic analogs of lantibiotics have been produced by chemically modifying lantibiotics (107, 108). The most common chemical modification of lantibiotics is through reactions with a free C-terminal carboxyl group. NVB302, is one such variant that has been chemically modified to have a diaminoheptane tail. This analog of actagardine has passed phase I clinical trials (113). Until the isolation of the mu1140-COOH analog, the presence of an AviCys residue on the epidermin group of lantibiotics prevented further development of novel analogs. The addition of a chlorinated aromatic ring may confer additional characteristics to mutacin 1140, as is seen in vancomycin analogs in which the vancomycin analog inhibited transglycosylase activity (184). Additionally, we have been able to conjugate a diaminoheptane tail on mutacin 1140, similar to NVB302. All of these analogs were bioactive, however further characterization of these analogs are still needed to determine whether they improve the compounds activity or stability in animal studies.

Previously, visualization of the bioactivity by the epidermin group of lantibiotics was limited to *in vitro* assays due to limitations of not being able to conjugate a fluorescent probe to the antibiotic (52). The addition of a fluorescein label to mutacin

1140, for the first time, allowed for the *in vivo* visualization of this class of lantibiotic in action. These data have provided new insights into the importance of decarboxylation for bioactivity of the epidermin group of lantibiotics. Class 1 lantibiotics are known to bind to lipid II by forming a cage around the pyrophosphate residue using rings A and B (the lipid II binding domain) (154, 185). Furthermore, the latter half of the peptide is believed to help in the lateral assembly of the lantibiotic-lipid II complexes to form islands (51, 186). In nisin, these islands form a pore complex, but the epidermin group primarily sequesters lipid II without forming a pore, as has been reported in fluorescently labeled lipid II vesicle experiments (50, 51). Decarboxylation was thought to be important, primarily, for stability of the peptide by preventing carboxypeptidases from degrading the lantibiotic (187). The loss of activity in the mu1140-COOH analog is intriguing and is likely to be the result of various factors, but our data suggests that the loss in activity is attributed, primarily, to loss in lateral assembly function. Microscopy studies show that mu1140-COOH can competitively bind to lipid II against nisin. Nisin and mutacin 1140 have been shown not to interact with each other, indicating that the decrease in fluorescence by the fluorescein labeled nisin analog was due to competition with the lipid II target (52). Additionally, the mu1140-COOH analog was shown to have a protective function against wild-type mutacin 1140 and nisin. This suggests that the lateral assembly activity is crucial for bactericidal activity and the presence of the C-terminal carboxyl group prevents mutacin 1140 forming a stable lipid II complex.

The influence of dehydrations and lanthionine ring formations on lantibiotic biosynthesis is well known (102, 163). Yet, little is known on how other PTMs influence

the biosynthesis of a functional lantibiotic. Studies have suggested that other PTM modifications, such as the N-terminal lactate of epilancin 15x, act independently of the dehydration and cyclase modifications found in lantibiotics(30, 87, 188, 189). Previous data has suggested that decarboxylation must occur before terminal ring formation due to a reactive ene-thiol intermediate that promotes terminal ring formation (177). Analysis of MrsD crystal structure has suggested that its active site cannot accommodate a lanthionine ring (176). Furthermore, attempts at decarboxylation of a lanthionine mimic proved futile (175). The lack of *in vitro* decarboxylation of mu1140-COOH shows that decarboxylation must occur before ring D formation suggesting that MutD cannot accommodate the terminal lanthionine ring into its active site. Additionally, the isolation of the fully modified mu1140-COOH analog demonstrates that terminal ring formation can occur regardless of the presence of a carboxyl group.

During *in vivo* synthesis of mutacin 1140, mutations that prevent ring formation or dehydrations within the lantibiotic will have unpredicted affects on other PTMs within the peptide (32). A better understanding of the role of each PTM will promote the synthesis of novel analogs. We demonstrated that the bioactivity of the mu1140-COOH variant can be restored by capping the C-terminus with an amine (Table 4.1). The chemical synthesis of the AviCys residue is cumbersome (190, 191). Our data demonstrates that chemical synthesis of this residue is not necessary for the epidermin group of lantibiotics and that solid phase peptide synthesis (SPPS) with differentially protected lanthionine can be used to synthesize this class of lantibiotics. Our study is a significant contribution to the field and provides new possibilities to synthesize novel

analogs of the epidermin group of lantibiotics. Moreover, our study has provided new insight into the importance of decarboxylation for bioactivity.

IV.5. Materials and methods

IV.5.1 *Bacterial strains and media*

The bacterial strains and plasmids used in this study are outlined in table 4.4. *S. mutans* strains, *B. subtilis* PY79, *S. pneumoniae,* and *M. luteus* ATCC 10240 were grown in either THyex media agar (30g/L Todd Hewitt Broth, 3g/L yeast extract, 15g/L agar; Bacto, Sparks, MD), THyex broth ((30g/L Todd Hewitt Broth, 3g/L yeast extract), or THyex top agar (30g/L Todd Hewitt Broth, 3g/L yeast extract, 7.5g/L agar; Bacto, Sparks, MD). *E. coli* was grown in LB medium (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl, pH adjusted with NaOH to pH 7.5), Terrific Broth (12 g/L tryptone, 24 g/L yeast extract, 4 mL/L glycerol, and 2.2g/L KH_2PO_4 and 9.4g/LK_2HPO_4), or LB plates (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl, 15g/L agar, and pH adjusted with NaOH to pH 7.5).

Table 4.4 Strains and plasmids used in chapter IV.

Strain	Plasmid intermediate	Relevant characteristic	Reference or Source
S. mutans JH1140 (ATCC 55676)		WT bacteriocin producing strain	Strain (124), ATCC
S. mutans Δ*mutA*		*mutA* deletion strain	(32)
S. mutans IFDC2:*mutD*		*mutD* gene replacement with IFDC2 cassette	This study
S. mutans Δ*mutD*	pΔ*mutD*	Clean deletion of *mutD*	This study
M. luteus ATCC 10240		Indicator strain for activity	Strain (40)
B. subtilis PY79		Indicator strain for activity	Strain (195)

Table 4.4 Continued

Strain	Plasmid intermediate	Relevant characteristic	Reference or Source
S. pneumoniae ATCC 27336		Indicator strain for activity	ATCC
E. coli DH5α	pCR2.1 and pET28B(+)	Intermediate cloning host	Invitrogen
E. coli BL21		Protein overexpression host	Invitrogen
E. coli p*mutD*-kan	p*mutD*-kan	Codon optimized *mutD*	This study
E. coli pet28B:*mutD*	pet28B:*mutD*	MutD overexpression	This study

IV.5.2 *Gene deletion of* MutD

Primers used for both sequencing and mutagenesis were designed using the *S. mutans* genome and *lan* cluster (GenBank/EMBL accession number AF051560). The IFDC2 gene replacement system was used, as previously described, to mutate or delete MutD(139). In-frame deletion cassette (IFDC2) is a gene replacement cassette containing a both a positive selection marker (*ermAM*) and a negative selection marker (-*pheS*)*. Approximately 500 bp upstream of *mutD* was amplified, and to prevent polar effects of downstream genes of *mutD*, a 500 bp fragment was amplified starting at 100 bp upstream of the stop codon and 400 bp downstream of *mutD*. The fragments were attached to the 5' and 3' end of the IFDC2 cassette, respectively, by PCR. The final 3kb fragment was transformed into *S. mutans* ATCC 55676 by competent stimulating peptide (CSP) protocol (141). An overnight culture of *S. mutans* ATCC 55676 was diluted to 0.1 OD_{600} and incubated at 37 °C to 0.25 OD^{600}. Two µl of 10µg/mL solution of CSP was then added to 200 µl of the 0.25 OD culture. After a 30 minute incubation time at 37 °C, 1µl of the PCR amplified product was added to the culture. The transformation was incubated at 37 °C for 5 hours before plating 50 µl of a 1000-fold

dilution onto a THyex plate containing 15µg/mL of erythromycin. Transformants were confirmed by PCR. The PCR products were inserted into a Topo PCR2.1® plasmid and were sent for sequencing. Both upstream and downstream regions were joined together and amplified to create the Δ*mutD* fragment. The Δ*mutD* fragment was transformed into *S. mutans* IFDC2:*mutD*, and selected on THyex plates (containing 4mg/mL of P-Chloro-phenylalanine).

Table 4.5 Primers used in chapter IV.

Primer	Sequence (5' to 3')	Characteristic
MutD-UpF	GAT TTG TTT CGT AAA GAG GGT TC	*mutD* gene replacement
MutD-DnR	CTA CAT CAA TCC CAG AAT CAA C	*mutD* gene replacement
MutD-UpR-IDH	GAGTGTTATTGTTGCTCGGAAATTATTTCTCCGTTCAG TTAA	*mutD* gene replacement
MutD-DnF-erm	GGTATACTACTGACAGCTTCGGTAATTGTTGGACAAGAATC	*mutD* gene replacement
DelMutD-F	TTAACTGAACGGAGAAATAATTGGTAATTGTTGGACAAGAATC	*mutD* clean deletion
DelMutD-R	GATTCTTGTCCAACAATTACCAATTATTTCTCC GTTCAGTTAA	*mutD* clean deletion

IV.5.3 *Bioactivity assays*

The deferred antagonism assay was performed as previously described (34). *S. mutans* strains were grown overnight in THyex broth at 37 °C. The cultures were diluted to 0.1 OD OD_{600} and grown to mid-logarithmic phase before diluting to 0.05 OD_{600}. Then, 2µl of the cultures were spotted on THyex plates in duplicates of triplicates. The wild-type *S. mutans* JH1140 and *S. mutans ΔmutA* were used as positive and negative controls for activity, respectively. The plates were incubated for 18 hours in a candle jar at 37 °C. After incubation, the strains were heat killed at 65 °C for 90 minutes. Fresh *M. luteus* grown overnight at 37 °C on THyex plates were used to inoculate pre-warmed THyex broth. The culture was grown to 0.6 to 0.8 OD before diluting to 0.2 OD_{600}. The culture was further diluted 25-fold in melted (42 °C) THyex top agar. Approximately 5mL of the top agar solution was spread on the heat killed bioassay plates and allowed to cool for 10 minutes. The plates were the place in the incubator (at 37 °C) for 18 hours.

The MICs were determined according to previously published protocol (40). A stock solution of the antibiotics tested was first suspended in 50% acetonitrile (ACN) at a concentration 640 µg/mL. This stock was subsequently diluted 2-fold until a final concentration 0.156 µg/mL was reached. Subsequently, 10ul of each dilution was placed into a well on a 96 well microtiter plate. *M. luteus* ATCC 10240, *S. pneumoniae*, and *B. subtilis* PY79 were grown overnight in THyex at 37 °C. Cultures were diluted in the morning to 0.1 OD_{600} and allowed to grow to 0.6 OD_{600}. This culture was diluted a 100-fold in fresh THyex media and then 400ul of this culture was added to 10mL of fresh

THyex. The suspension contains approximately 10^5 colony forming units (CFUs). The bacterial suspension (190 µL) was added to each well containing 10 µL of antibiotic suspension or solvent blank. This results in another 20-fold dilutions of the antibiotic suspension. For the competition assays, the 10^5 CFU bacterial suspension was initially mixed with mu1140-COOH for 15 minutes at the 1X MIC of either mutacin 1140 (0.25 µg/mL and 0.125 µg/mL for *B. subtilis* and *M. luteus*, respectively) or nisin (0.5 µg/mL for *B. subtilis* and *M. luteus*). Following the 15 minute pretreatment, 190 µL was added to each well as described above. The MIC is described as the lowest concentration of antibiotic that prevented visible growth after 24 hours.

IV.5.4 *Production and purification of mutacin 1140 and mu1140-COOH*

Lanthipeptides isolated in this study were cultures as stated previously(192). *S. mutans* strains were grown in a modified THyex media. The media contained 30g/L Todd Hewitt, 3g/L yeast extract, 1 g/L NaH_2PO_4, 0.2 g/L Na_2HPO_4, 0.7 g/L $MgSO_4$, 0.005 g/L $FeSO_4$, 0.005 g/L $MnSO_4$, and 0.3% agar. The semi-solid agar (1 L) was inoculated with various strains of *S. mutans* and incubated at 37 °C for 72 hours. After incubation, the inoculum was frozen at -80 °C overnight and thawed for 1 hour in a 65 °C water bath. The inoculum was then centrifuged at 20,000 G for 30 minutes and the supernatant was collected. The supernatant was mixed with chloroform at a 1:1 ratio and shaken vigorously. This mixture was again centrifuged at 20,000 G for 30 minutes. The precipitate between both the aqueous and chloroform phases was collected and dried overnight. The dried product was resuspended in 35% ACN containing 0.1%

trifluoroacetic acid (TFA) and ran on either a semi-prep C18 column (Agilent® ZORBAX, ODS, C18, 5µm, 4.6x250mm) or analytical column. Peaks collected were confirmed by mass on a Shimadzu® MALDI-MS on both linear and reflectron modes.

IV.5.5 *Chemical modification of mu1140-COOH and nisin*

Labeling of C-terminal carboxyl group with methylamine (33% in EtOH) (Sigma-Aldrich), diaminoheptane (Sigma-Aldrich), 4-chlorobenzylamine (Sigma-Aldrich), 3,4-dichlorobenzylamine (Sigma-Aldrich), or 5-(aminoacetamido)fluorescein (Sigma-Aldrich) was done by 1-Hydroxy-7-azabenzotriazole/1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (HOAt/EDC) coupling. The labeling was done in accordance to a previously described method(34, 51). The reaction mixture was suspended in 100 µl of dimethyl formamide (DMF) with 50 nmols of either nisin, or mu1140-COOH, 50 nmols AAA-fluorescein or 200 nmols of the primary amine, 60 nmols of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), and 60 nmols of 1-hydroxy-7-azabenzotriazole (HOAt). The reaction was covered in foil and incubated at room temperature for approximately 16 hours. The reaction was subsequently diluted 10-fold with 35% ACN containing 0.1% TFA and ran on an analytical C-18 column. The labeled peptides were confirmed by MALDI-MS as described above. The amount of chemically conjugated product was determined by Bradford Assay using manufacturer protocols (Sigma-Aldrich). The purified labeled peptides were then dried down and resuspended in 35% ACN containing 0.1% TFA at a concentration of 100ug/mL and stored at -20 °C.

IV.5.6 *Microscopy*

B. subtilis PY79 was grown and treated with fluorescein labeled peptide as previously described (34). A fresh plate of *B. subtilis* was used to inoculate THyex broth and incubated approximately 16 hours at 37 °C. The culture was diluted 20-fold and place back in the incubator for 3 hours at 37 °C. Then, 100 µl of the culture was incubated with the antibiotic (10µg/mL) for 15 minutes. The cells were pelleted and resuspended in 100 µl of phosphate buffer solution (PBS). The wash step was repeated three times before fixing with 1.6% formaldehyde in PBS. After fixation the cells were washed with PBS three more times and the remaining pellet was suspended in 50 µL of PBS. The sample (30 µL) was added to a slide and observed using an Olympus confocal microscope with a 100×/0.90 dry objective. A 488 nm argon laser was used to excite the fluorophore. For the competition assay, the bacterial culture was initially incubated with mu1140-COOH or native mutacin 1140 at a concentration of 10 µg/mL for 15 minutes, washed, and resuspended in fresh media. 10 µg/mL of fluorescein labeled nisin was then added for 15 minutes, before following the wash protocol previously stated.

IV.5.7 *Mu1140 double labeling and Edman sequencing*

Edman sequencing has been frequently used to determine the sequence of small peptides, such as lantibiotics (193, 194). Mu1140-COOH was doubly labeled as previously described for mutacin 1140 (29). A 200 µM solution of Mu1140-COOH in 5µl of water was added to a reaction tube containing 2 mg of sodium borohydride. Then, 94µL of solution B (570 mg guanidine HCl, 100 mL N-ethylmorpholine and water

to a final volume of 1 mL; the pH of the mixture was adjusted to 8.5 with glacial acetic acid) was added to the reaction mixture and placed into a glass vial. The reaction vial was purged with nitrogen and stored at 37 °C for three days. The peptide was then loaded onto a prosorb column (Applied Biosystems) and absorbed onto a PVDF membrane. After drying the PVDF membrane, 15 ul of solution A (280 µL methanol, 200 µL water, 65 µL 5 M sodium hydroxide, 60 µL ethanethiol) was added to the membrane. The reaction was sealed tightly and incubated at 50 °C for 1 hour. After the reaction, the sample was sent out for Edman sequencing. The glass fiber filter used in the Edman sequencing was pretreated with polybrene to reduce the loss of peptide per cycle. After drying in nitrogen, the PVDF membrane was excised and loaded onto a sequencer (Applied Biosystems 492 Protein P.E. Biosystems, Foster City, CA, USA). The sequence was analyzed by the ABI 610A data software. D,L-2-aminobutyric acid was commercially purchased from Sigma Aldrich and used as a standard.

IV.5.8 *MutD cloning and purification*

A codon optimized sequence of *mutD* for *E. coli* was purchased from Integrated DNA Technologies (IDT, Coralville, IA). According to manufacturer's specifications, the *mutD* gene was cloned into the Xho1 site within the pET28B(+) expression vector (EMD Millipore, Billerica MA) providing an N-terminal His-tag. The ligation was transformed into *E. coli* DH5α and confirmed by sequencing. The plasmid was then transformed into the *E. coli* BL21 expression strain. A fresh plate of *E. coli* BL21 pET28B(+):*mutD* was restreaked onto LB plate containing 50 µg/mL kanamycin (kan)

prior to induction. One colony from the plate was suspended in 1 L of Terrific broth containing 50 µg/mL kan. Before the addition of IPTG (250ul of a 1M solution), the culture was shaken at 37 °C until an OD_{600} of 0.8 was reached. Following induction, the culture was incubated with shaking at 18 °C for approximately 16 hours. The culture was then spun at 4000 x g for 30 minutes at 4 °C. The pellets were resuspended in 25mL of lysis buffer (500mM NaCl, 50mM Tris-HCl, 15 mM imidazole, 1mM PMSF, 10% glycerol, at pH 7.5), before adding 500µl of the lysozyme solution (50mg/mL). The solution was mixed and stored on ice for 30min. The suspension was lysed using a sonicator at medium setting for 10 minutes with 1 minute intervals, taking care to not overheat the solution. The lysate was then centrifuged at 16,000 x g, and the supernatant was collected. 500 µl of Ni-NTA beads were added to the supernatant and placed on a shaker for approximately 16 hours at 4 °C. The Ni-NTA beads were collected by centrifugation at 3,000 RPM for 10 minutes at 4 °C. The beads were washed three times with 10x bead volume of lysis wash buffer (500 mM NaCl, 50 mM Tris-HCl, 30 mM imidazole, 1mM PMSF, at pH 7.5). After washing, the beads were eluted by resuspending in 500 µL of lysis buffer containing 0.5 M Imidazole. The suspension was place on a shaker for 1 hour at 4 °C and the elution was repeated three times. The elutions were run on an SDS Page gel to determine purity of the MutD. The deacarboxylase was further run on an FPLC. Protein concentrations were determined by Bradford assay (Sigma-Aldrich).

IV.5.9 *In vitro decarboxylation*

In vivo decarboxylation was performed as previously described by Kupke et al (175). A control substrate, SFNSYTC was purchased from Peptide&Elephants. A 1 mg/mL solution of either SFNSYTC or mu1140-COOH in Tris-HCl buffer (pH 8.0) containing 3mM DTT was prepared. The peptide solution (100 µL) was incubated with MutD (30 µg/mL) for 1 to 10 hours at 37 °C. The sample was diluted 10-fold in 35% ACN containing 0.1% TFA before being loaded on the RP-HPLC as previously described(40). The masses of the isolated fractions were determined by MALDI-MS.

IV.5.10 *Lipid II binding assay*

Lipid II was a kind gift from Eefjan Breukink and was resuspended in a 1:1 Methanol:Chloroform solution. The lipid II binding assay using thin layer chromatography (TLC) was done as previously described (34). The mobile solvent consisted of consisted of butanol:acetic acid:water:pyridine (15:3:12:10 [vol/vol/vol/vol]). A 0.2 mM solution of mutacin 1140 or mu1140-COOH in 10 µL of solution A was mixed with lipid II (final 6.8 mM) for 1 hour in a sealed glass vial. This corresponded to a ratio 3:10 peptide:lipid II ratio. All of the reaction mixtures and the appropriate controls were spotted (5 µL) 2 cm from the bottom of the plate. These spots define the origin of the plate. Lipid II and peptide alone were used as a control to demonstrate that the origins do not stain unless peptide and lipid II are added together. The mobile phase was allowed to climb up the plate until it reached a centimeter from the top. The plate was allowed to dry before staining with iodine.

CHAPTER VI:

CONCLUSION

Ever since the discovery of penicillin, the threat of antibiotic resistance has been a concern for public health officials and scientists across the world. In modern times, this threat has become an ever-concerning reality. Public health has become increasingly threatened by multi-drug resistant bacteria. The term "superbug" is increasingly heard in news articles, which have conveyed a sense of an upcoming antibiotic apocalypse (1). Yet, the real cost of these pathogenic bacteria is hard to ignore. MRSA has become one of the leading causes of hospital deaths across the world (118). Furthermore, the naturally antibiotic resistant spore-forming bacteria, *C. difficile*, has become one of the most persistent hospital acquired infections (118). Even vancomycin, which is known as a drug of last resort, is becoming increasingly susceptible to antibiotic resistant organisms (125). Healthcare systems have seen an increase in healthcare spending due to these infections (2, 3). These pathogens have put an immense burden on healthcare systems in the world. As such, the CDC and other public health agencies have identified ways to combat this problem. The development of novel types of antibiotics has become one of the key tools in combating this problem. Bacteriocins, like mutacin 1140 and other lantibiotics, may assist in alleviating the burden on healthcare.

Lantibiotics have become an enticing candidate for development due to their unique structure, target, and lack of antibiotic resistance (9, 17). Development of mutacin 1140 and other lantibiotics requires an understanding of the mechanism of biosynthesis. The key components of lantibiotic biosynthesis are known and much

research has been done on them. Dehydrated serines and threonines are important, especially ones that are involved in lanthionine ring formations. The thioether linkages found in lanthionine rings are absolutely important for activity and stability of the bacteriocin (32, 34, 163). The PTM enzymes themselves have also been studied extensively. Crystal structures and mechanism of actions for the dehydratase, NisB, and the cyclase, NisC have been elucidated (102, 103). Furthermore, LanM, found in class II lantibiotics, has been shown to have enough freedom to modify other peptides (43, 44). LanP, is similar to other serine proteases. While the transporter, LanT, is similar to other ABC-type transporters. The immunity genes found in lantibiotics are composed of a 2-component histidine kinase system. Even though lantibiotics may share similar core peptide structures and PTM enzymes, the coordination or PTM modifications, transport and regulation are divergent.

Mutacin 1140, as a member of the epidermin class of lantibiotics has several shared structural elements found within the core peptide (171). It has a similar lipid II binding domain to epidermin and nisin, but its lateral assembly domain is distinct from nisin (171). The C-terminus of the lateral assembly domain of the epidermin class is capped to form a C-terminal AviCys residue. The mutacin 1140 leader peptide is structurally unique compared to the epidermin class of lantibiotics. As a therapeutic, mutacin 1140 has shown tremendous promise. It has been shown to be active against various Gram-positive pathogens in *in vitro* assays. It has been shown to clear infections in murine models (14). Yet it is plagued with the same problems that other lantibiotics have faced, which is the low fermentation yields found in this type of bacteriocins and

the short half-life in animals (14, 36). That is why it is imperative to study the biosynthesis of lantibiotics, such as mutacin 1140, to help in its development.

Our studies have shown that the unique structure of the mutacin 1140 leader peptide is important for biosynthesis. Mutacin 1140 has a 61 amino acid leader peptide, which is longer than other lantibiotic leader peptides with a similar core peptide structure. Deletions of the N-terminal part of the leader peptide have suggested that length not the secondary structure is important for N-terminal portion of the leader peptide. This lack of sequence specificity in portions of the leader peptide is similar to the nisin leader peptide (127). The lack of specificity is in contrast with the class II lantibiotic, lacticin 481, which shows that proline insertions do affect the production of the lantibiotic (123). Protein structure predictions of mutacin 1140 suggest that there may be a certain preference of amino acid sequence in the leader peptide which do not sterically hinder binding to the target (24). The presence of a specific four amino acid motif has been shown to be important in the biosynthesis of most lantibiotics. The EDLF motif in mutacin 1140 is unique compared to other class I lantibiotics which contain the FNLD box (123, 128). Products obtained from mutants in the EDLF box suggest that the leader peptide binds to a single site on one of the PTM enzymes. Binding studies on the nisin leader peptide suggest the FNLD box is important for binding to the dehydratase, NisB (127). Our model suggest that the length of the leader peptide is important for initial binding and stabilizing interactions with presumably the MutB dehydratase, while the EDLF motif is essential for anchoring the leader peptide to a defined site within the dehydratase. Having a defined binding site within the leader peptide and the dehydratase

is important for coordinating the PTM modifications on the core peptide ensuring that the PTM modifications are occurring in the correct order enabling the synthesis and transport of a functional bacteriocin.

Proteolytic cleavage of the leader peptide is an important step in the production of lantibiotics. It has always been assumed that a single proteolytic cleavage is required for lantibiotics. The specificity of the protease in removing the leader peptide is not well understood. Some models suggest that the transporter may help guide the core peptide to the protease. Yet deletion of the transporter, MutT, did not change the activity or mass of the product isolated. Mutations in the nisin core peptide suggest that the core peptide itself governs cleavage at the normal site (117, 153, 165). Our data suggest that this may also be the case, wherein the removal of rings A and B, by alanine substitution at cysteine positions seven and eleven, had a product with eight amino acids of the leader peptide attached. Furthermore, a single ring A mutant produced a mutant with a cleavage site found internally of the core peptide. The presence of an 8 amino acid leader peptide to mutacin 1140 in a ΔMutP mutant suggests that another proteolytic cleavage occurs. Studies by Rink *et al* have shown that partial cleavage of the leader peptide does occur in strains without NisP, yet the major product includes the whole leader peptide (162). In contrast, the deletion of MutP clearly shows a single partially cleave product, suggesting a different sort of mechanism for partial cleavage. The secondary cleavage site is position specific, with the cleavage occurring at various regions of the leader or core peptide depending on the mutations made at the C-terminal end of the leader peptide. A deletion of the amino acids (-7 to -2) positions shifted the cleavage site into the core

peptide. This observation suggests coordination between the different PTM enzymes. Our model proposes that MutB and MutC activity is coordinated by structural dynamics of the modified core peptide, wherein full modification would trigger interaction with the transporter and release of the core peptide from MutB by the secondary cleavage site at the -8 position. Our model suggests that the last PTM modification would be the formation of ring D, which is essential for the transport of the core peptide.

One model of lantibiotic biosynthesis suggests that cyclization and dehydration occur independently of each other (126). Another models of biosynthesis challenges this model and suggest that PTM dehydration and cyclization modifications occur concurrently by binding of the leader peptide to a single enzyme (127). Our data on the dehydration and cyclization of the lanthione rings advocates the latter model of biosynthesis. Mutations on some of the rings resulted in the lack of dehydrations on some of the serines and threonines at other positions within the core peptide, which suggests coordination between these two PTM modifications. Interestingly, one minor product also included a variant that had a carboxyl group on the C-terminus, which has never been isolated in this class of lantibiotics. The mechanism for knowing how PTM system recognizes a fully modified peptide for transport has not been elucidated. The lack of any product from a ring D mutant suggests that ring D formation, which is assumed to be the last PTM modification, is the deciding factor for transport outside the cell. Preventing the formation of both rings C and D still yielded significant levels of transported product. This further gives credence to the PTM coordination model of lantibiotic biosynthesis where the leader peptide provides an anchor for the core peptide

modifications, in lieu of the movement of the leader peptide between PTM enzymes for subsequent modifications (123).

Although lantibiotic biosynthesis models and studies have mainly focused on the primary forms of modification, understanding other types of PTM modifications is still important. Lantibiotics contain numerous post-translational modifications, each with varying degrees of importance to the activity and biosynthesis of the bacteriocin. The importance of the AviCys residue in mutacin 1140 and other epidermin class lantibiotics is unknown. The crystal structure of the decarboxylase and the proposed mechanism of formation of the AviCys have been elucidated, but their role in bioactivity has not been elucidated until our study (137, 176). Cross species production of EpiA have suggested that decarboxylation is important for preventing proteolytic degradation of the bacteriocin (187). The isolation of a carboxyl variant of mutacin 1140 is the first instance of an epidermin class of lantibiotic with a carboxyl group at the C-terminus. Furthermore, this variant contains all the modifications except for the decarboxylation, demonstrating that decarboxylation is not important for the full biosynthesis of the peptide. The study also supports the notion that decarboxylation would have to occur before ring D formation, which we suggest is important for transport. The lack of activity of this analog shows that decarboxylation is crucial for activity. The presence of a negative charge at the C-terminus in physiological conditions may affect lateral assembly of the lantibiotic-lipid II complex. Binding data to lipid II shows that the mutacin–COOH can still bind to lipid II, which shows that presence of a carboxyl group prevents lateral assembly of the complex. This observation has been found in nisin,

wherein an intact lipid II domain did not have activity. Interestingly, similar to nisin AB fragment, mu1140–COOH bound to lipid II at a lower affinity. Previous studies have suggested that the lateral assembly domain is important for stabilizing the binding to lipid II. My research shows decarboxylation may not be important for full biosynthesis, but it is absolutely necessary for the antibacterial activity of mutacin 1140.

The presence of the AviCys residue has always posed a challenge for the development of the epidermin class of lantibiotics. This has hampered chemical synthesis of the epidermin class of lantibiotics by adding a cumbersome step for synthetic synthesis (190. 191). Due to low yields of lantibiotics by naturally producing organisms, solid-phase synthesis of lantibiotics has become an attractive option for production. Unfortunately, the AviCys residue prevents chemical modification of this class to produce novel C-terminal analogs. C-terminal modification of lantibiotics, such as nisin, has produced novel analogs that have additional characteristics (105-107). Our production of novel analogs of mutacin 1140 by chemically modifying the C-terminus of mu1140-COOH solves this problem. Furthermore, restoration of activity shows that producing the AviCys residue is not needed, but simply capping the C-terminus restores activity. A variant of deoxyactagardine B, NVB302, with a diaminoheptane tail has gone through phase I clinical trials (113). The addition of a diaminoheptane tail on the end of mutacin 1140 restores activity, yet further studies are needed to evaluate how this analog may affect its application for treating a bacterial infection. The formation of a carboxyl variant or mutacin 1140 will promote new drug development studies and further the

potential for chemically synthesizing active analogs of the epidermin class of lantibiotics.

The competing models of lantibiotic biosynthesis has shown that further studies are needed to fully understand PTMs of lantibiotics. Our studies have shown that lantibiotic biosynthesis follows the coordinated PTM model. Wherein, the leader peptide acts as an anchor on MutB while the dehydratase and cyclase modify the peptide in a coordinated manner. Decarboxylation of MutD is assumed to occur before ring D formation. Finally formation of ring D signals the peptide to be transported out of the cell, and subsequent leader peptide cleavage produces the active peptide antibiotic. Although there is a clearer picture in mutacin 1140 biosynthesis, there are still many challenges ahead in producing mutacin 1140 as a therapeutic. The low yields by lantibiotics still pose a problem in scaling up production. Yet our recent data has shown that by manipulating the mutacin 1140 regulatory pathway, the production of the lantibiotic can be increased. Furthermore, mutacin 1140 has a short half-life *in vivo* (ref), the synthesis of new variants of mutacin 1140 by chemically modifying the peptide could solve this problem. Due to its broad spectrum of activity against Gram-positive bacteria and the lack of resistance of pathogenic bacteria to mutacin 1140, supports the need for additional studies aimed at furthering its use to treat infections. The mutacin-COOH variant is a great scaffold for producing novel analogs by coupling the C-terminus. Mutacin 1140 and other lantibiotics have shown promise inthe fight against antibiotic resistance. Research into these class of bacteriocins has grown over the years, yet much needs to be done.

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